EFFECTS OF PHYSICAL FACTORS ON THE GROWTH OF *MONASCUS PURPUREUS* WENT

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CHAPTER ONE

INTRODUCTION

The genus Monascus was erected by van Tieghem referring to two fungi growing on a boiled potato culture in France in 1884 (van Tieghem, 1884). These fungi aroused popular attention in the beginning of this century due to their widespread distribution on cereal grains, starch, silage, etc., and great economic lost of storage food and poisoning of horses was also reported(Buchanan, 1910). Growth of Monascus species on ruber and in soil has also been observed(Dayal and Giri, 1968; Schade, 1937).

It is evident that Monascus is a cosmopolitan genus. But this genus is supposed to be of Asian or European origin and brought to other continents together with crops transplanting from one continent to another like corn(Young, 1930).

The morphology and development of the cleistothecia in Monascus extensively attracted some investigators and different interpretations were put forward(Barker, 1903; Kuyper, 1905; Ikeno, 1903; Olive, 1905; Schikorra, 1909; Young, 1931). Barker suggested that sexual reproduction occur in this genus and the development be just the same as what we know of Ascomycetes. Ikeno suggested that free cell formation take place leaving epiplasm. Now, Barker's suggestion is adopted and the taxonomic status of Monascus is given as follows(Bessey, 1965) :

Class : Ascomyceteae

Order : Aspergillales

Family : Aspergillaceae

Genus : Monascus

Generally, the vegetative hyphae range from 4 µm to 6 µm in width, but those producing fruiting bodies are shorter and more slender(Young, 1931). Hyphae considerably branch in a monopodial or pseudo-dichotomous manner. As a type of asexual reproduction, conidia are generally produced, ovoid to pyriform in shape and vary considerably in size from 6 x 5 µm to 16 x 14 µm. The conidia are borned singly or produced in basipetal chains of 2 to 6 or even more. They are sometimes tinged with red in old cultures; however, they are usually colorless. The cleistothecia develop in great numbers upon the hyphae and are generally terminal, though sometimes apparently lateral(Buchanan, 1910). Cleistothecia are usually spherical 25 to 55 µm in diameter, or subspherical 37 x 36 µm to 50 x 42 µm, walls colorless to light reddish brown. Ascospores are ellipsoidal 5 x 4 to 6.5 x 4 µm, colorless to pink colored(Young, 1930). The ascocarp was formerly called the perithecium, but the rationale is not known. Actually the ascocarp is entirely closed without any ostiole, so the writer recommends to use the term cleistothecium instead of perithecium. Although a cleistothecial mutant could sometimes evolve from the perithecial fungus (Maniotis, 1965), this may not be the case for Monascus.

Van Tieghem described two species, viz. M. ruber, the type form and M. mucoroides(van Tieghem, 1884). In 1895, Went described

M. purpureus which he obtained in Java, but this species was originally grown in China (Went, 1895). Afterwards, a number of new species and varieties were added to this genus. They are : M. albidus Sato, M. albidus var. glaber Sato, M. anka Nakazawa et Sato, M. anka var. rubellus Sato, M. araneous Sato, M. barkeri Dangeard, M. bisporus (Fraser) von Arx, M. fuliginosus Sato, M. heterosporus (Harz) Schroter, M. major Sato, M. olei Piedallu, M. paxii Lingelshein, M. pilosus Sato, M. pubigerus Sato, M. rubiginosus Sato, M. rubropunctatus Sato, M. serorubescens Sato, M. serotenus Sato, and M. vitreus Sato (Manandhar and Apinis,1971; Young, 1930; etc.).

Most of the species are microthermophilic, growing in a temperature range of 18 to 45 C with optima at 30 to 38 C, but a few which may be considered as transitional mesophiles are unable to grow at 40 C (Manandhar and Apinis, 1971).

In studying the conidia formation of Monascus ruber and a highly identical species Backusia terricola, Cole and Kendrick erected a new form genus, Basipetospora. They described conidium formation in this new genus as follows :"The first conidia are derived from a restricted meristematic zone which appears immediately below the preceding conidium. As the chain of conidia extends, the conidiophore becomes shorter"(Cole and Kendrick, 1968).

Monascus purpureus Went is characterized particularly by having ascospores which are usually spherical, being 5 µm in diameter, or slightly ovoid, their size being 6 x 5 µm. The youngest part of the mycelium is white, but it rapidly changes to a rich pick and later to carmine red (Young, 1930).

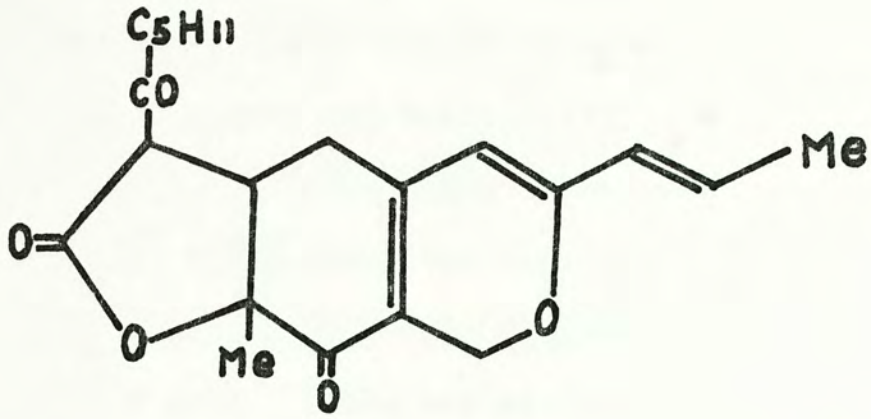
As mentioned before, the morphological studies of Monascus had once been the subjects of investigations by some mycologists in the beginning of twentieth century, but precise and authenic reports using modern technique and photography are still lacking. On the other hand, the physiological studies of this genus are no better than the morphological parts.

M. purpureus grows more rapidly on invert sugar than on either glucose or fructose. The addition of monosaccharides can increase the utilization of sucrose, and fructose is more efficient than glucose in such activation process(Lilly and Barnett, 1962). The Monascus species utilize starch so efficiently that they are given the name of "starch fungi". M. serorubecens was shown to produce large amount of glucoamylase and specially efficient strains were selected(Ho et al., 1973). Saito studied the enzymes of M. purpureus and found that in Czapek-Dox media supplemented with 5 % D-glucose zinc uptake by M. purpureus was correlated with an increase in both phosphate and D-glucose uptake, increased growth, and increased production of carbon dioxide and ethyl alcohol. Added zinc also increased growth and reduced the economic coefficient when either arabinose, xylose, glucose, fructose, sucrose, maltose, starch, or ethyl alcohol was the sole carbon source. A particular combination of amino acids(glycine, L-leucine, and L-tryptophan) added to the media also stimulated growth to an equal or much greater extent than did the addition of zinc(Johnson and McHan, 1975; McHan and Johnson, 1970). They suggested that zinc may involve in regulatory processes integrating carbohydrate and nitrogen metabolism during

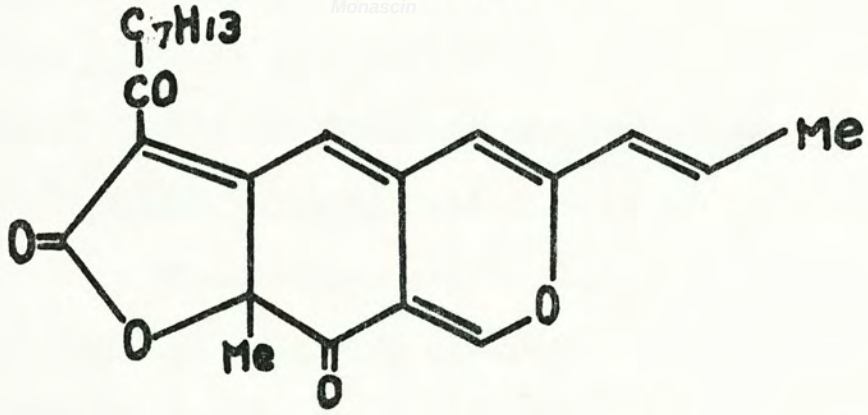
growth.

The pigmentation of M. purpureus and related species has been investigated by some mycologists and chemists. There are mainly two kinds of pigments, the red and the yellow. The red pigment, monascorubrin, was first isolated by Nishikawa in 1932 from M. purpureus and the molecular structure was determined(Fielding et al., 1960). The yellow pigment was also isolated by Nishikawa, and he asigned the name monascoflavin(Nishikawa, 1932). Now this pigment is generally called monascin and its molecular structure was first proposed by Fielding et al.(Fielding et al., 1961) and confirmed by Chen and his colleagues in 1971(Chen et al., 1971). Another pigment rubropunctatin was isolated by Haws and his group from M. rubropunctatus (Haws et al., 1959), and it was also separated from the extracts of M. purpureus(Hadfield et al., 1967). Some of the chemical natures of these pigments were revealed, and some derivatives were also worked out from these pigments, ascorubramine and apomonascorubramine from monascorubrin, and rubropunctatamine, aporubropunctatamine, O-methyl-aporubropunctatamine and O-acetyl-aporubropunctatamine from rubropunctatin(Fielding et al., 1960). The oxidation of monascin will give rubropunctatin(Chen et al., 1971). The structures of monascorubrin, monascin and rubropunctatin are shown in Fig. 1-1 (Turner, 1971).

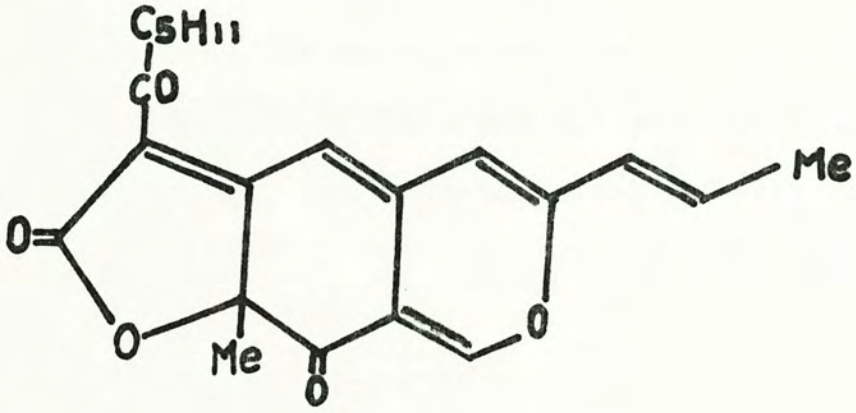
M. purpureus and some of the related species are rather famous and have been used in different ways in China and some of the oriental countries. The red-mold rice which is also known as ang-kak, anka, ben-koji, aga-koji, Chinese red rice and red rice, is made



Monascin



Monascorubrin



Rubropunctatin

Fig. 1-1 : The structures of monascin, monascorubrin and rubropunctatin.

by the growth of the fungus M. purpureus in glutinous rice. The red-mold rice has been traditionally used in Chinese medicine with wide range of applications(Bau and Mo, 1975). In the southeastern part of China and Taiwan, this fungus is used for the manufacture of an alcoholic liquor(Sato and Naito, 1935). "Red rice wine", "Glutinous rice wine" and "Cheng Kang Chiew" can be easily purchased from market now. "Red preserved bean curd"(Red Chinese cheese) is also a popular Chinese food, made by growing of the fungus on the surface of bean curd. Since the ability of these fungi to digest starch is admirably good, they are used in the glucose manufacturing industry(Ho et al., 1973).

The present study involves the effects of physical factors on the growth of Monascus purpureus and some of its related aspects. In order to make a clear presentation, this report is divided into few chapters under the following headings : comparative study on the germination of conidia and ascospores, effects of laser, X-ray and fast-neutron on the germination of conidia and ascospores, morphological studies on the mutants derived from ionizing radiation treatments, effects of visible and ultraviolet light on pigmentation and sporulation, the anti-bacterial activity and pigmentation of this fungus.

CHAPTER TWO

COMPARATIVE STUDIES ON THE CONIDIUM AND ASCOSPORE GERMINATION OF *MONASCUS PURPUREUS* WENT

Introduction

As in other microorganisms, spores are important stages in fungal life cycles. The asexual cycle composes of asexual spore germination, vegetative growth and sporulation. On the other hand, sexual cycle is similar in stages, but complicated with nuclei fusion and probably genetic exchange. In Ascomycetes, reproduction is characterized by the asexual conidium and the sexual ascospore (Alexopoulos, 1962).

Generally, germination is defined as the transformation of a relatively inactive spore to the highly active vegetative thallus(Schmit and Brody, 1976). Biochemical as well as morphological changes take place in the accomplishment of the germination processes, and the morphological changes are constantly used as criteria to differentiate the germinated and those dormant or dead spores(Allen, 1965; Florance et al., 1972; Goldstein and Erb, 1972; Khan, 1975; Schmit and Brody, 1976). Some investigators consider the spores as germinated if germ tubes equal in length to the width of the spores are produced(Dubin and English, 1975; Manners, 1966). Others define germination as the protrusion of the germ tube from the spore wall (Cother and Griffin, 1974). On some special occasions, the product-

ion of definite vesicles (Celerin and Fergus, 1971; Verma and Petrie, 1975) and prominant swelling (Goldstein and Erb, 1972) are considered as criteria.

The study of spore germination covers different types of spores, namely, conidium (Blakeman, 1975; Fletcher and Morton, 1970; Martin and Nicolas, 1970), chlamydospore (Cother and Griffin, 1974), oospore (Verma and Petrie, 1975), trichospore (El-Buni and Lichtwardt, 1976), ascospores (Celerin and Fergus, 1971; Paden, 1974), uredospore (Chang et al., 1974; Marchetti et al., 1976) and basidiospore (Hansen and Patton, 1975; Morton and French, 1974) etc. The fungal species studied range extensively broad, for example, the saprophytic fungi like Penicillium (Gottlieb and Tripathi, 1968) and Aspergillus (Pass and Griffin, 1972); the plant pathogenic fungi like Puccinia (Calpouzos and Chang, 1971), Phytophthora (Banihashemic and Mitchell, 1976) and Albugo (Verma and Petrie, 1975); animal pathogenic fungi like Sumittium (El-Buni and Lichtwardt, 1976); mycorrhizal fungi like Glomus (Green et al., 1976) etc. However, the spore germination study of Monascus species is neglected. Though a number of sexual and asexual spore germinations have been studied on different species, no comparative study of both spores of the same species is hitherto presented. This may be due to the difficulty in getting both spores from the same species. Fortunately, Monascus purpureus can be easily induced to produce handsome amount of conidia and ascospores, therefore a comparative study is possible. Since this fungus is widely distributed and causes damage to cereals and starch, the study of spore germination may provide some knowledge about the protection of

food stuff against this fungus in addition to the mycological interests.

Materials and Methods

A. Origin of fungus

M. purpureus was isolated from red-mold rice purchased from market. Using single spore isolation technique, the fungus was cultured for several changes on malt extract agar(Table 2-1) or potato-dextrose-agar. A wild-type identical to that described by Young(Young, 1930) was isolated and stored within a screw caped bottle on malt extract agar and used as stock culture.

B. Culture of fungus

Throughout this study, the fungus was cultured on malt extract medium in liquid state or solidified with 1 % agar(Table 2-1). Other media like water agar, Czapek-Dox medium were also used in some cases (Table 2-1). All the media were adjusted to pH 5.5 with 1 N NaOH and 1 N HCl before autoclaving. All the media were autoclaved at 121 C for 15 min. With 1.5 x 10 cm test tubes, agar slants were prepared and inoculated with the stock fungus and incubated at 35 C in total darkness. At the first two days, the fungal colonies were white to pale yellow colored and later on rapidly changed to deep red color. Fifty ml of liquid medium was dispensed into 250 ml flask. After inoculation, the flasks were incubated in a New Brunswick shaker-incubator at 35 C and at 200 rpm to provide enough aeration. The cultures were also kept in total darkness. The young

colony was white colored and globose shaped, it changed to red color at the end of day 3.

Table 2-1 : Composition of some media.

(A) Malt extract medium		
Peptone	10	g
Malt extract	5	g
Glucose	20	g
Distilled water	1000	ml
(B) Czapek-Dox medium		
$NaNO_3$	2.0	g
KH_2PO_4 or K_2HPO_4	1.0	g
KCl	0.5	g
$MgSO_4 \cdot 7H_2O$	0.5	g
$FeSO_4 \cdot 7H_2O$	0.01	g
Glucose	20	g
Distilled water	1000	ml
(C) Water agar		
Deionized water	1000	ml
Agar	10	g

C. Isolation of spores

Being cultured in malt extract liquid medium, M. purpureus produced tremendous amount of conidia, so all the conidia used in this germination study were provided from liquid culture. In studies other than the influence of age, conidia from 4-day culture were used. The whole culture was filtered through 4 layered and formerly

sterilized cheesecloth and passed through several centrifugation steps as shown in Fig. 2-1. Conidia obtained this way were quite pure and they were further washed three times with sterile distilled water. The final spore concentration was adjusted to 10^6 spores per ml with a haemacytometer. The spore suspension was stored at 4 C. Through the studies, the spore suspension was kept fresh and those kept at 4 C over three days were discarded.

On malt extract agar slant, M. purpureus produced a large number of cleistothecia which originated in day 3. Numerous ascospores were enclosed in each cleistothecium. Comparatively, conidia were sparse. A few ml of sterile distilled water were added to each 5-day culture in test tube and the culture was scratched with a glass rod. The effluent was filtered through 4 layered cheesecloth and the filtrate containing largely of ascospores and some fragments of hyphae were again filtered through Millipore glass-base and finally ascospores of high purity were obtained. The ascospores were also washed three times, adjusted to 10^6 spores per ml and stored at 4 C. Only fresh and stored less than 3 days spore suspensions were used.

D. Germination studies

The ordinary 2.5 x 7.5 cm slide glass as well as micro-culture slide were used. The slides were placed in 9-cm petri dish with crossed ridges on the bottom. All the glasswares used in the study were sterilized at 160 C for 2 h and cooled down before use. Two to three ml of sterilized distilled water were dropped into the bottom of the petri dish to keep moisture sufficient for the germination processes. Three drops of melted malt extract agar were dropped on the slide. Special media were also used in some occasions.

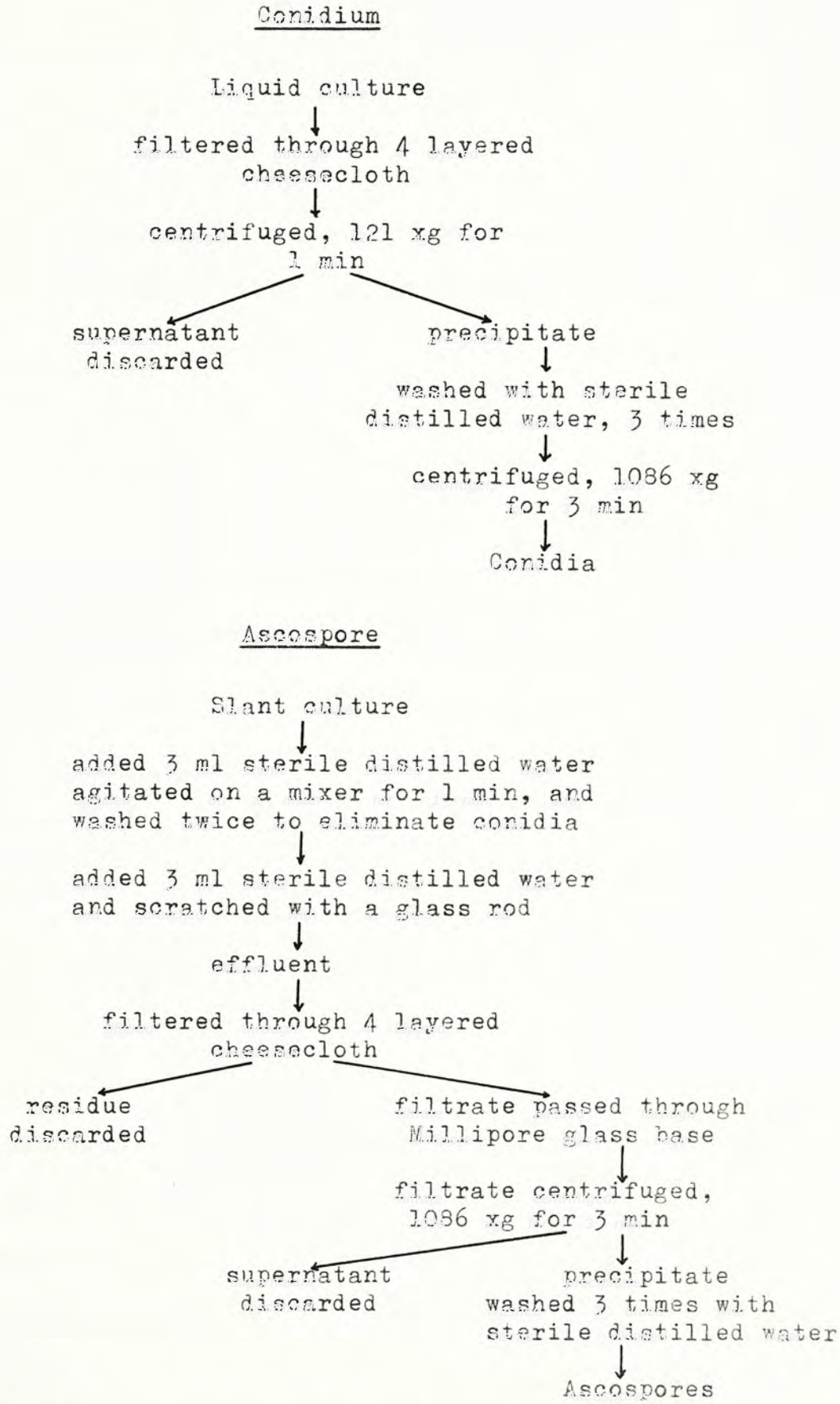


Fig. 2-1 : Isolation of spores.

With an inoculation loop, one loopfull of spore suspension of density 10^6 spores per ml was spread over the resolidified agar blocks. The conidia cultures were incubated at 35 C for 4 h, while the ascospore cultures were incubated for 8 h. During the counting, other cultures were kept at 4 C to stop further germination. The spore germinations were counted under light microscop at 200x magnification. Since the size of conidium is relatively large and germ tube can be easily discerned, so the conidium was considered as germinated when germ tube was apparently observed. While the ascospores are much smaller in size, only those spores with germ tubes equal in length to the broad side of the spores were considered as germinated. Percentage of germination was calculated as the ratio of germinated spores to the total spores and multiplied by hundred percent. All the experiments were replicated at least twice. Five fields from each agar block were counted. There were 3 agar blocks on each slide, and in total no less than 30 fields and 500 spores were scored. The mean value and the standard deviation were calculated and significance was also determined in appropriate cases.

Results

A. Morphology of germination

On malt extract agar, germination of conidia took place at a much faster speed than ascospores. At 35 C germination of conidia initiated half hour after incubation and the rate rose rapidly at one and half hour, appearing to be somewhat synchronous. A maximum of

about 95 % was reached, so the incubation period of 4 h was already enough for the study of conidium germination. The germination of ascospores began at hour 2 and the rate increased gradually. Evantually, a maximum germination percentage of 56 % was reached at the hour 8 (Fig. 2-2)

The protrusion of germ tubes through conidial wall was rather random and no special site was observed. Generally, only one germ tube was produced from each conidium. Two to 6 germ tubes from one conidium were also detected but the occurrance decreased with the increase of number of germ tubes(Plate 2-1). In the case of ascospore germination, one to two germ tubes were formed and protruded also randomly through the wall. There was no clear breaking of outer ascospore wall observed under light microscope. Some of the ascospore germinated to form a small vesicle and from which a germ tube was produced(Plate 2-2).

B. Effect of age

The age of the cultures from which the spores were isolated also affected the germination of the spores. Ascospores from different aged slant cultures were prepared and the germination was studied. The result is clearly shown in Fig. 2-3. Maxima were recorded on the 5- and 6-day cultures. Accompanying the aging of the culture, the germination percentage decreased smoothly and a decrease of approximately 10 % was recorded at the 10-day culture. In case of conidial germination, conidia from day 4, 5 and 6 malt extract liquid medium cultures had maximum germination percentage. The germination percentage decreased smoothly with a decrement of about 10 % at 10-day culture.

Plate 2-1 : Germination of conidia on malt extract agar. All the spores were stained with 0.1 % acid fuchsin and taken at 1000x. A, Ovoid shaped conidium with truncated end before germination. B, Initial of germ tube inside the conidial wall. C,D, Protruding through conidial wall. E, Conidium with 2 germ tubes. G, Conidium with 3 germ tubes, the third one is partially out of focus. H, Conidium with 4 germ tubes, note the septa formed separating the older germ tubes from the newly formed germ tubes, the fourth one is partially out of focus. I,J, Different planes to show conidium with 5 germ tubes. K, Conidium with 6 germ tubes. L,M, Branching of germ tubes after they has achieved certain growth. N, The wall of germ tube is continuous with the inner layer of conidial wall.

Plate 2-2 : Germination of ascospore on malt extract agar. Except A, all the ascospores were stained with 0.1 % acid fuchsin and all taken at 1000x. A, Ovoid ascospores before germination. B, Initial of germ tubes. C, Ascospore with single germ tube, note the branching. D,E, Ascospores with 2 germ tubes, but protruding through the wall at different sites. F, Branching of germ tube. G, Ascospore with one vesicle produced before the formation of elongated germ tube.

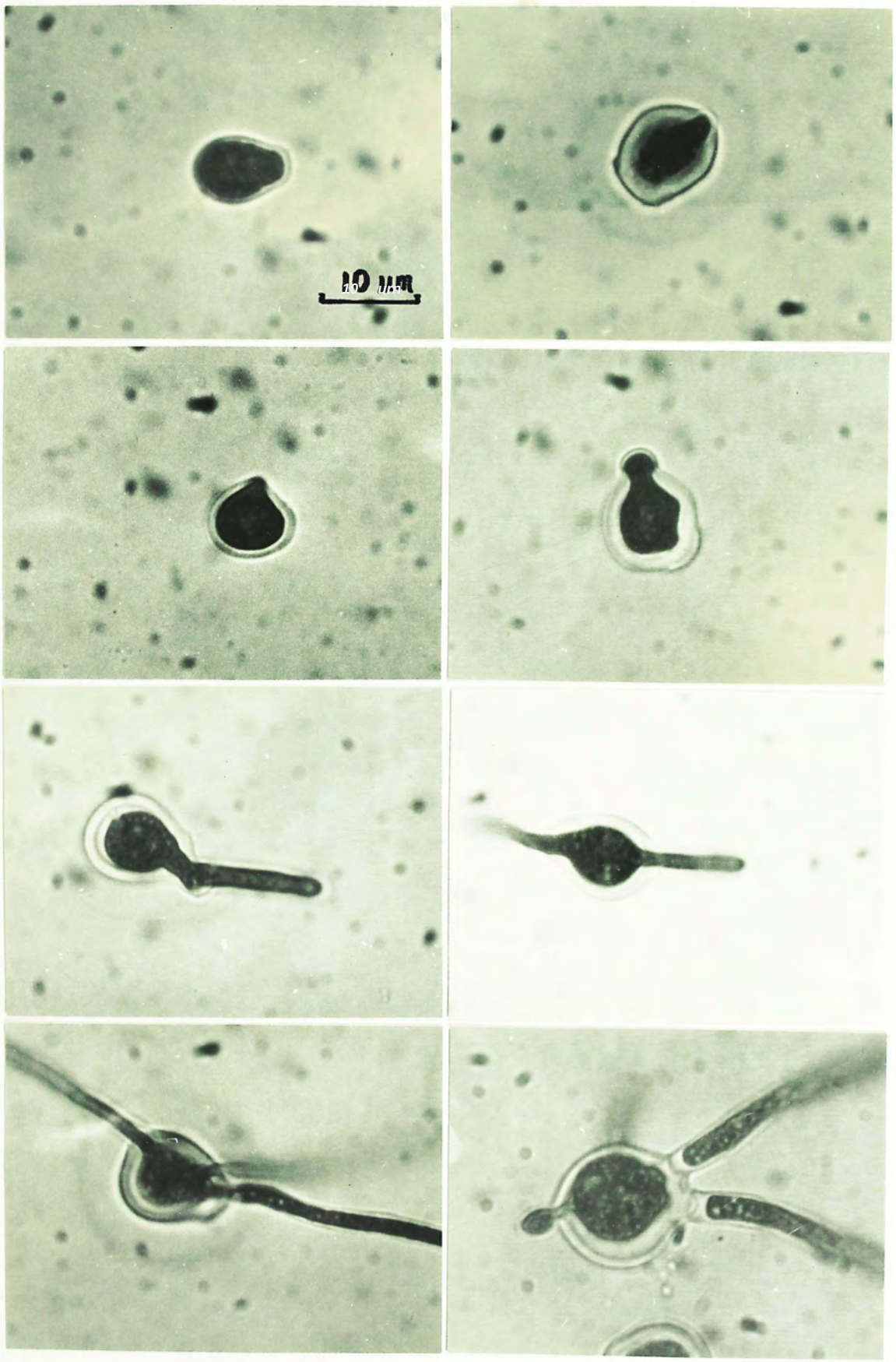
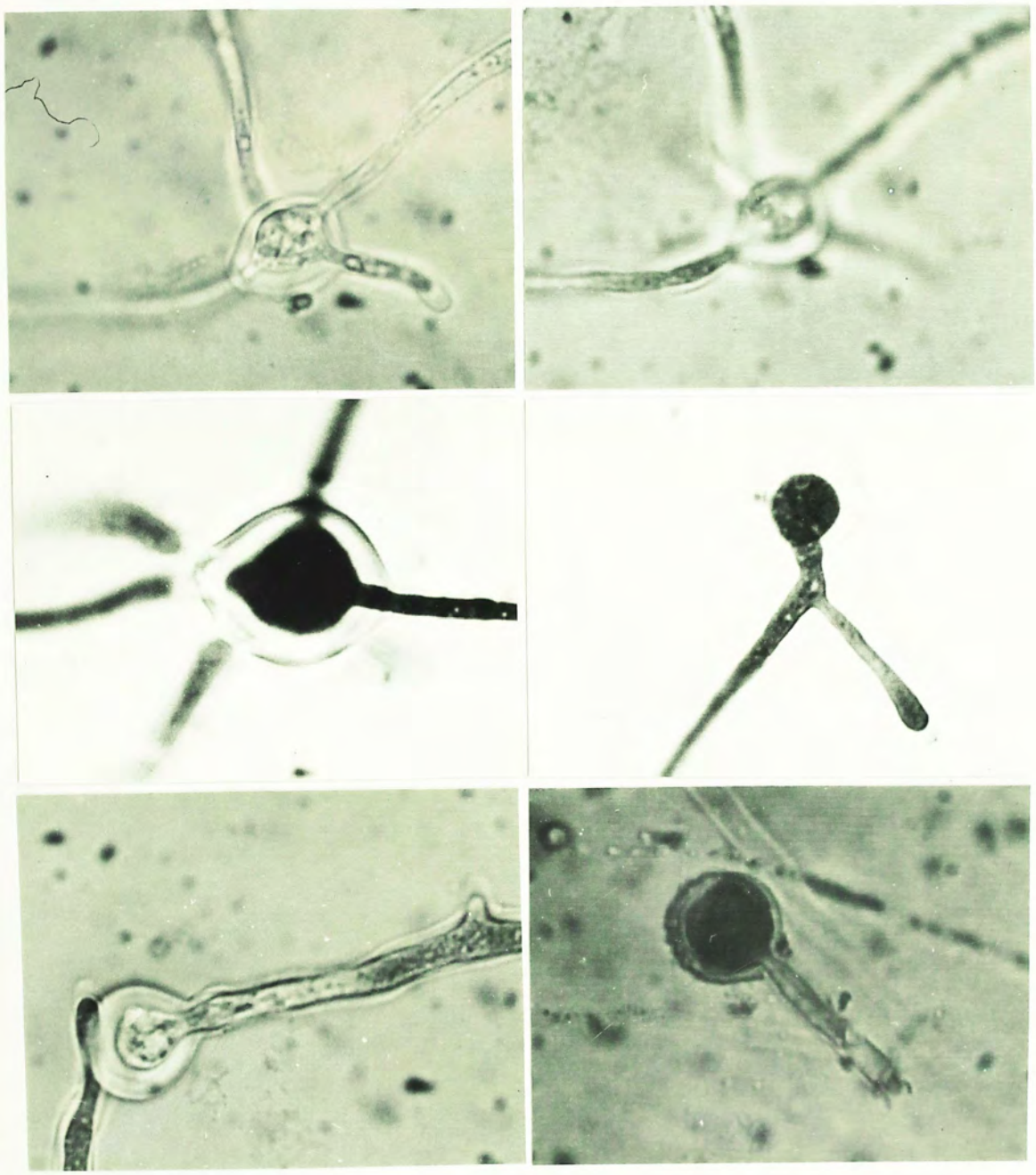


Plate 2-1

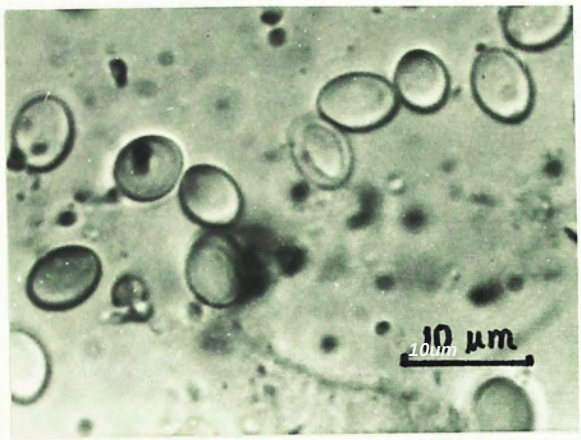


I J

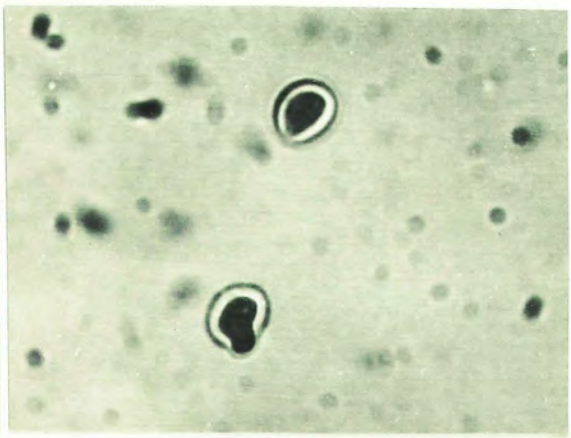
K L

M N

Plate 2-1 (Continued)



A



B

C

D

E

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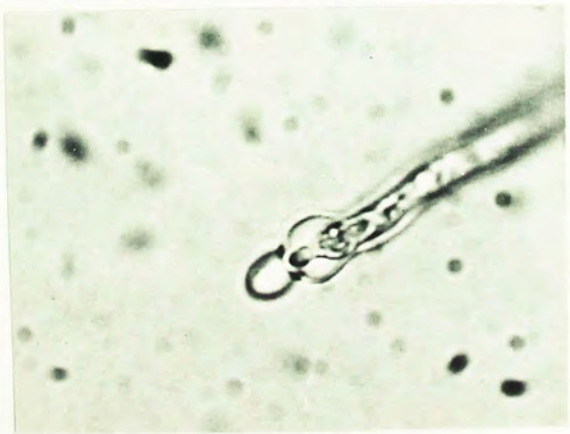


G

Plate 2-2

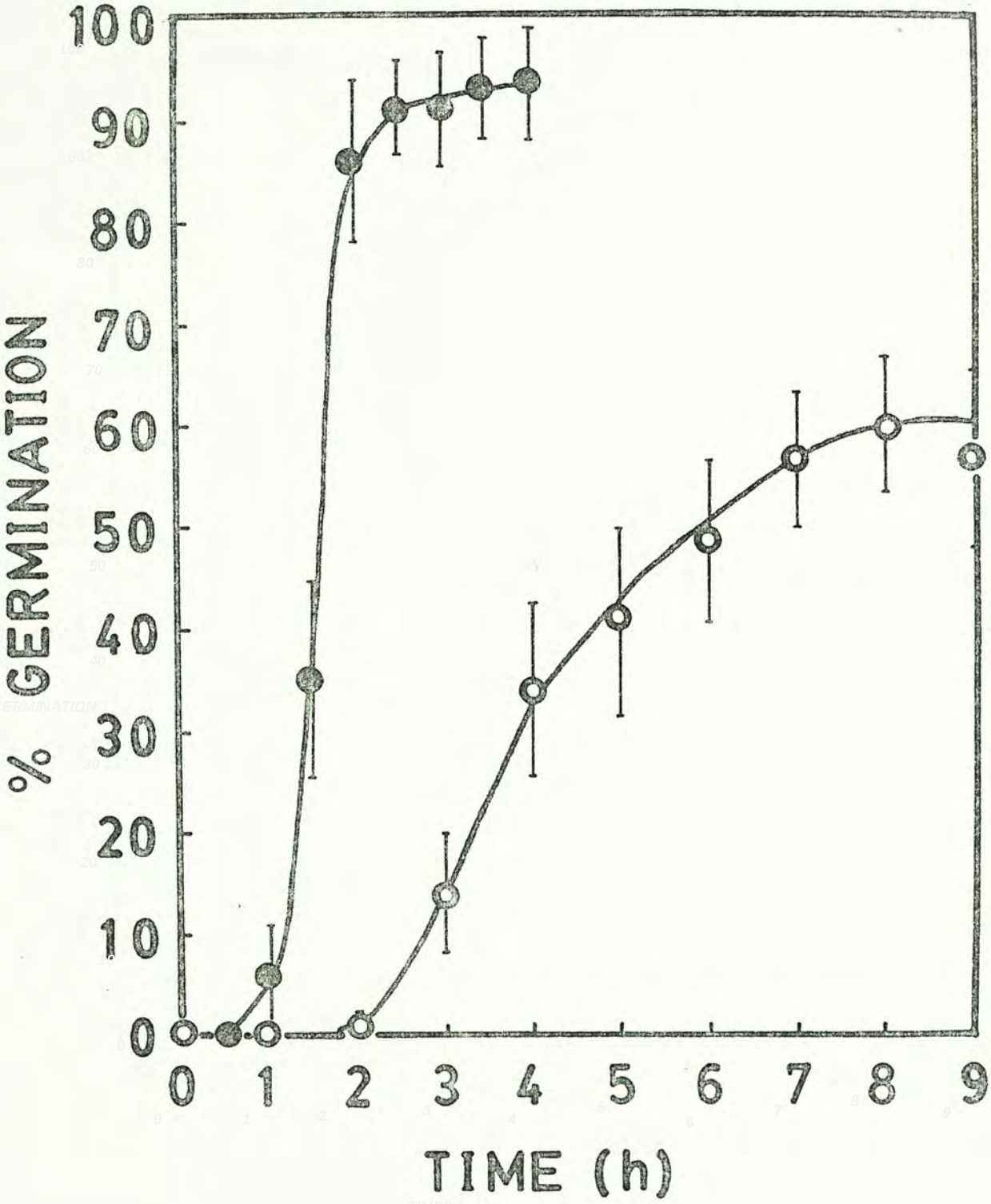


Fig. 2-2 : Time course of conidium(●) and ascospore(○) germination.

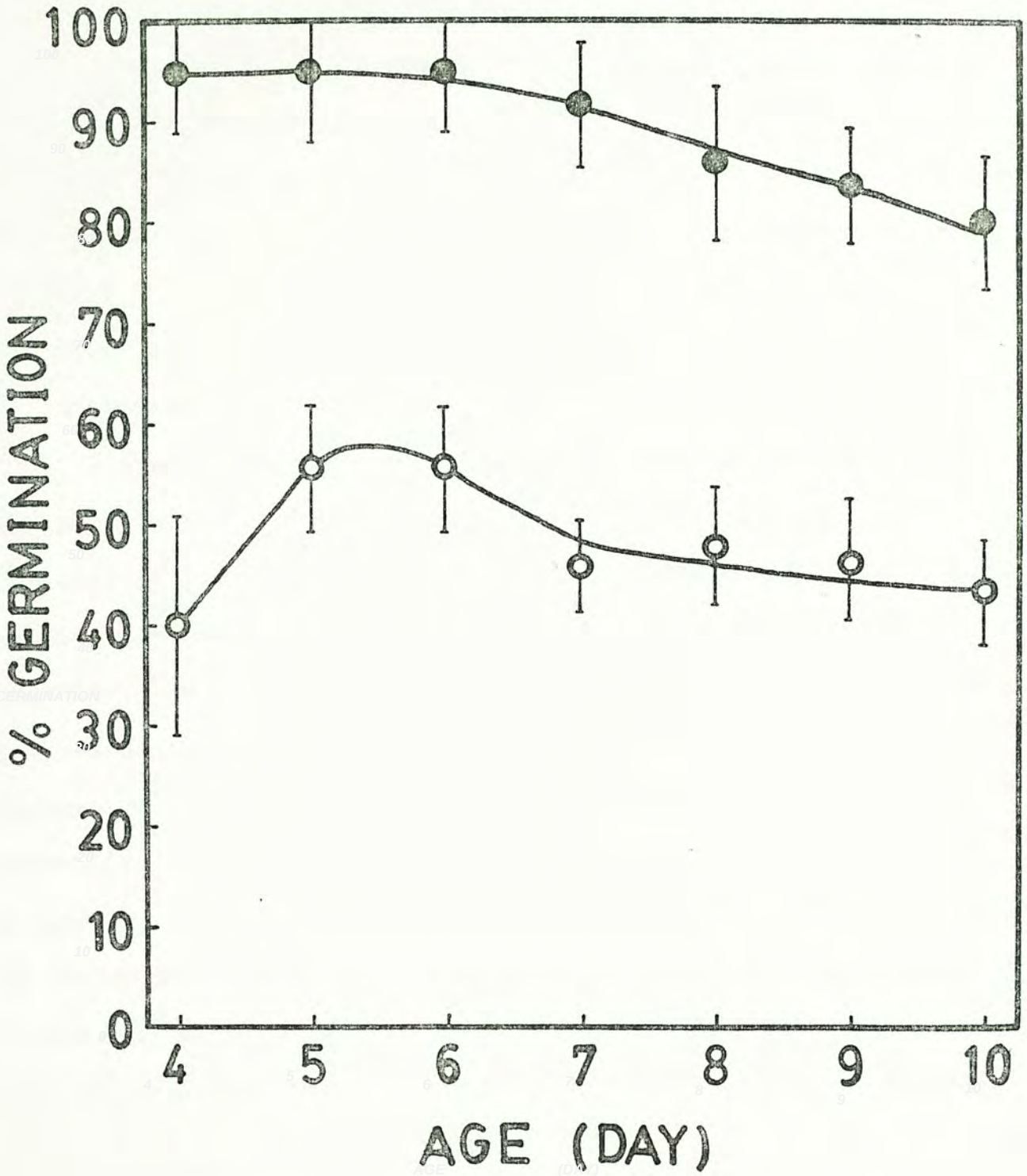


Fig. 2-3 : Effect of age on conidium(●) and ascospore(○) germination.

C. Effect of spore suspension density

Different spore suspension densities were tested. In both conidium and ascospore, the germination percentage did not decrease with the increase in spore density. The conidium density increased from 5×10^4 to 5×10^6 spores per ml, and the ascospore increased from 3.5×10^5 to 3×10^7 spores per ml. Actually augments were observed both in conidium and ascospore germination (Fig. 2-4).

D. Effect of pH

Citrate-phosphate buffer, 0.1 M(Colowick and Kaplan, 1955) was used in the study of concentration of hydrogen ion, i.e. pH on the germination percentage of conidia and ascospores. The buffer solution and the medium were autoclaved separately and then added together, the pH values were determined by a pH meter, type PHM29 of Radiometer Copenhagan. The results were shown in Fig. 2-5. The optimum pH for conidium germination was 4.8, pH 4 to 6 were also favorable, while pH values over 6 and below 3 were inhibitory with a decrease of more than 10 % at pH 2.6 and 20 % at pH 7. The optimum pH values for ascospore germination ranged from 2.6, the lowest value tested, to pH 5. Another buffer solution, acetate buffer, was further used in ascospore germination, the optimum value even extended to pH 6.

E. Effects of temperature

Incubation temperature greatly affected the germination of both conidium and ascospore. The spore cultures were incubated at five different temperatures, 25, 30, 35, 40, and 45 C, and the germination percentages were determined. It was apparently that

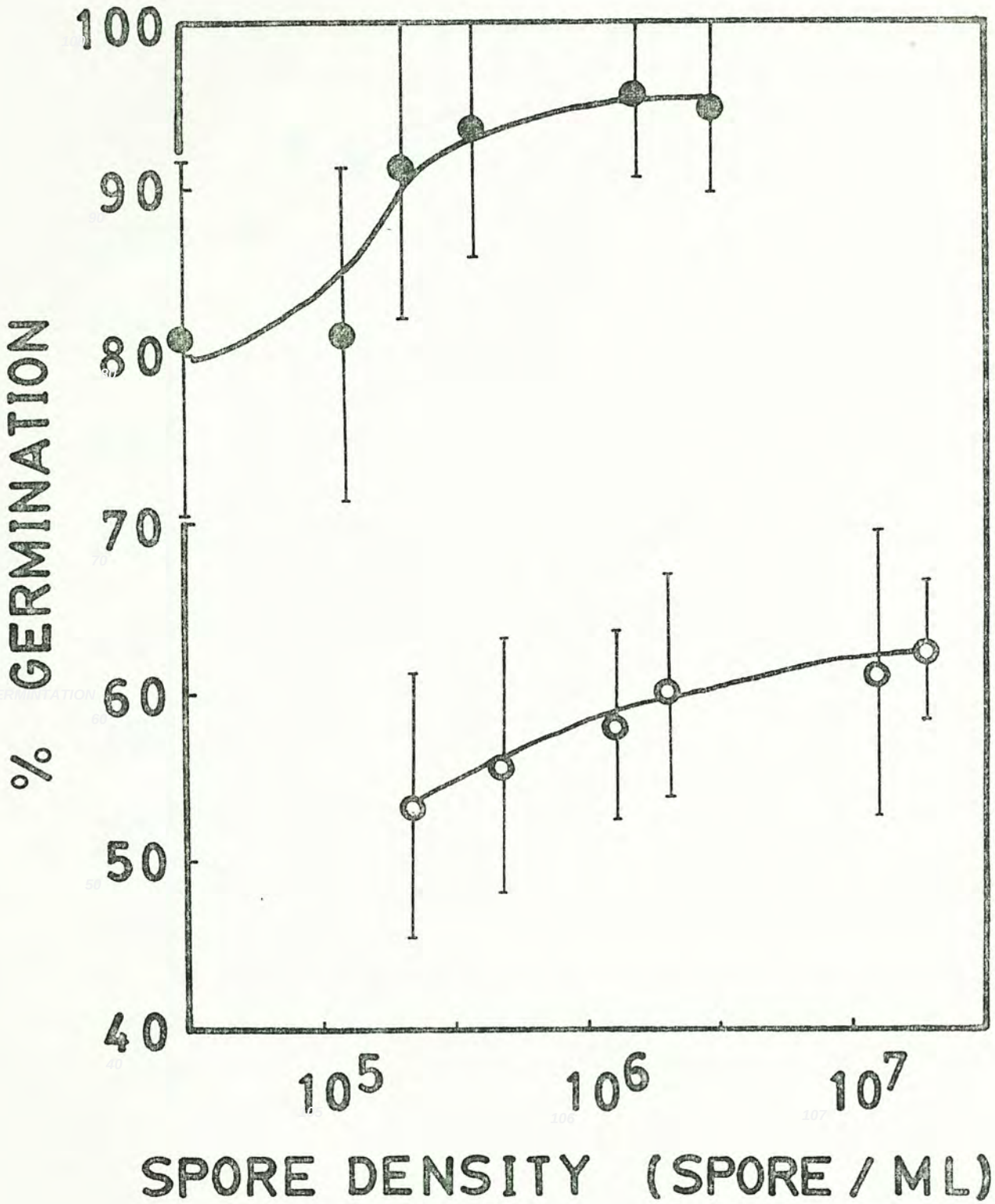


Fig. 2-4 : Effect of spore density on the germination of conidium (●) and ascospore(○).

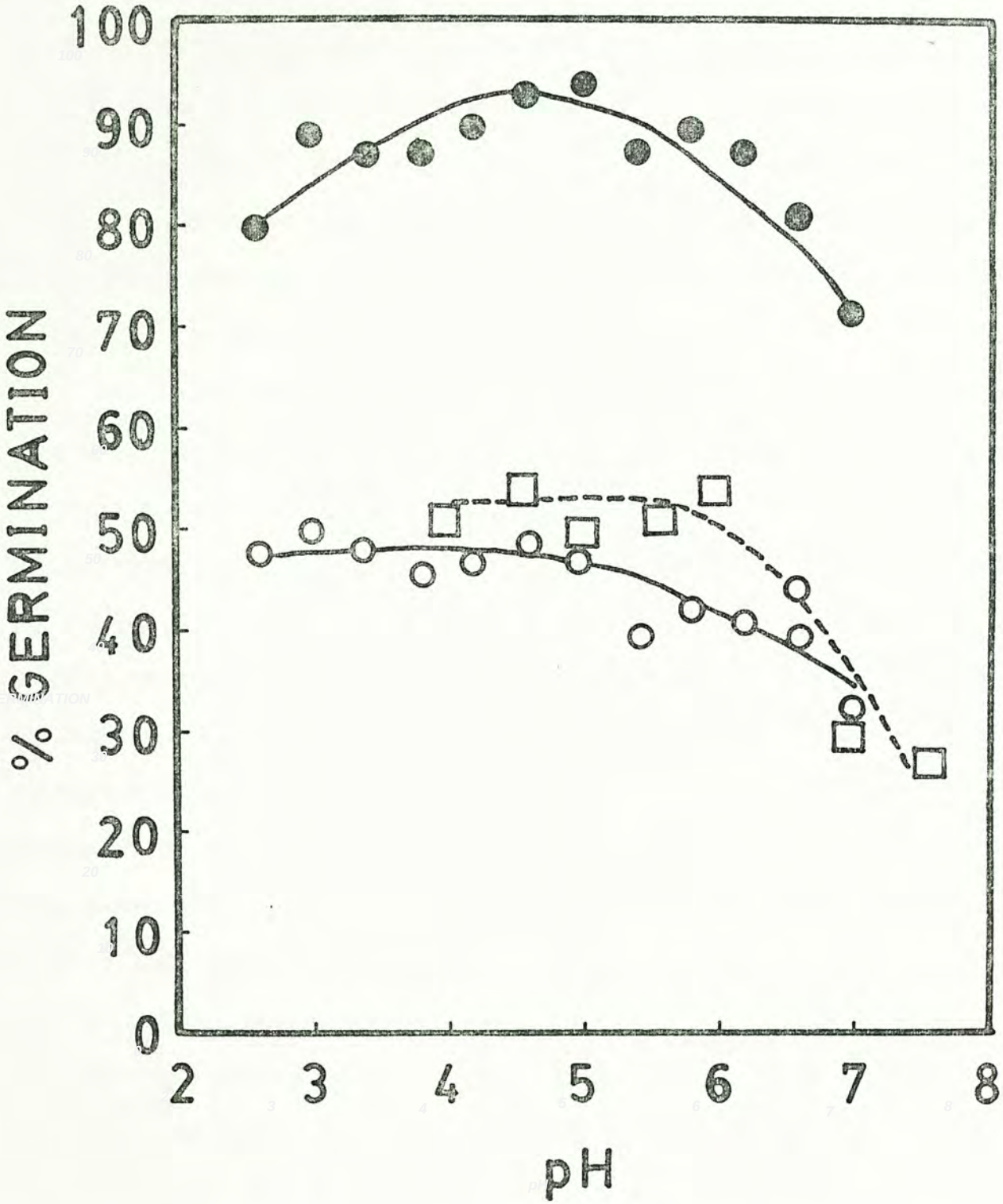


Fig. 2-5 : Effect of pH on conidium and ascospore germination. Solid circle, conidium; open circle and open square, ascospore; circle, 0.1 M citrate-phosphate buffer; square, 0.1 M acetate buffer.

conidium had a broader optimum range from 30 to 40 C than ascospore which had an optimum temperature at 35 C. The conidium germination percentage at 25 and 45 C dropped sharply from approximately 90 % to 37 %(Fig. 2-6). From Fig. 2-6, it was clearly shown that no such optimum temperature range existed in ascospore germination as it was in conidium germination. However, higher temperatures were somewhat detrimental to ascospore germination.

The high temperature resistancies of conidium and ascospore were also tested. To test high temperature resistancy, 1 ml of spore suspension was dispensed to each 1.5 x 10 cm thin-walled test tube and incubated in thermostatic water baths at 50 and 60 C for 5 to 30 min at 5 min intervals. After the heat treatment, spores were immediately inoculated to malt extract agar to check the germination percentage. The results were shown in Fig. 2-7. Conidium was more susceptible to high temperature, whereas 50 C could inhibit its germination and the severity enhanced with the increase in period of heat treatment, and 60 C treatment for even 5 min could completely inhibit the germination. Ascospore was much more heat resistant than conidium, whereas 50 C was only slightly affected the ascospore germination, but 60 C also inhibited the ascospore germination. Nevertheless there was still about 12 % germination detected after 60 C treatment for 30 min.

F. Effect of medium

The medium used in the spore germination tests was the natural complete malt extract agar. It is composed of carbon sources, growth factors such as amino acids and vitamins, and metallic ions.

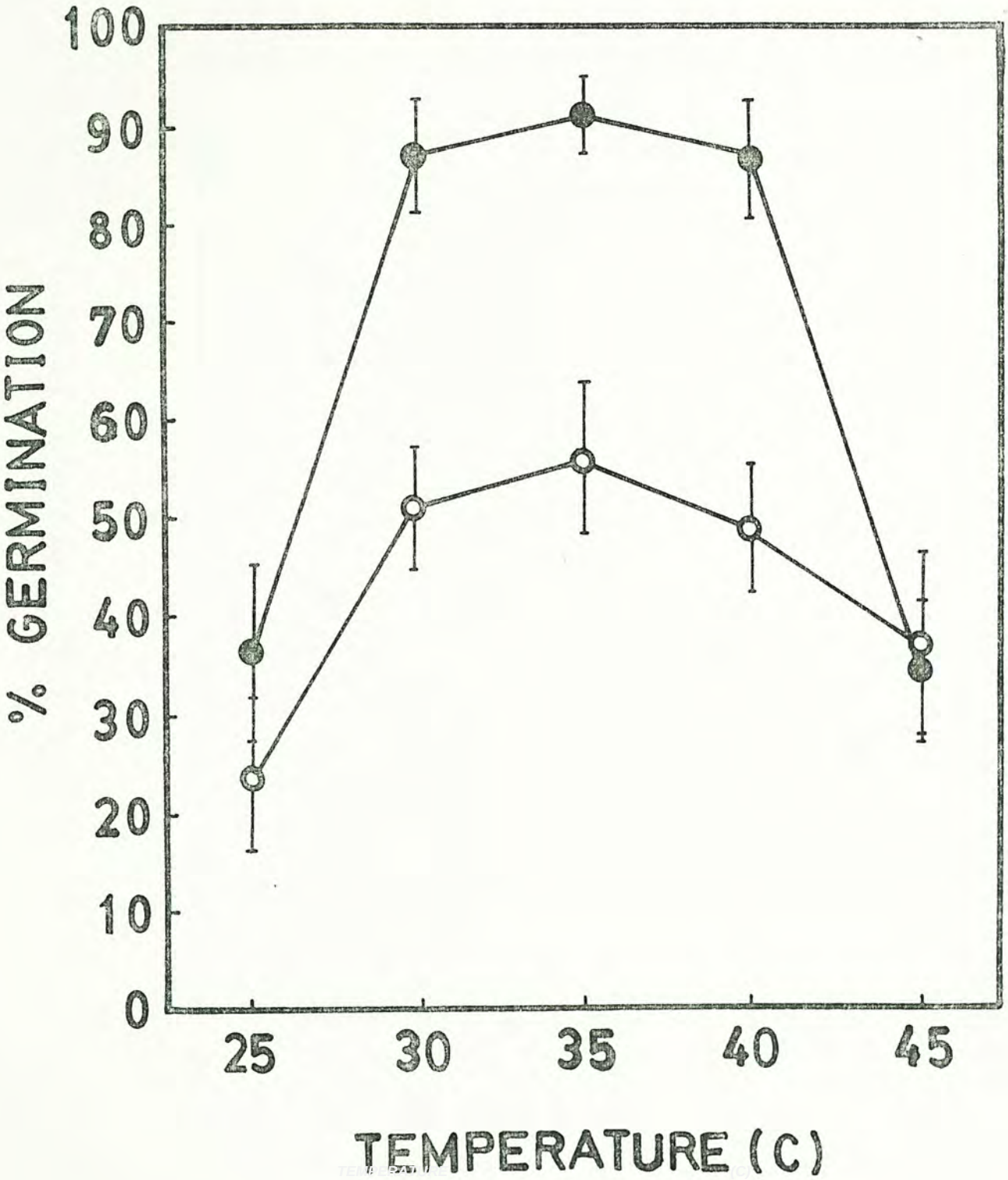


Fig. 2-6 : Effect of temperature on conidium(●) and ascospore (○) germination.

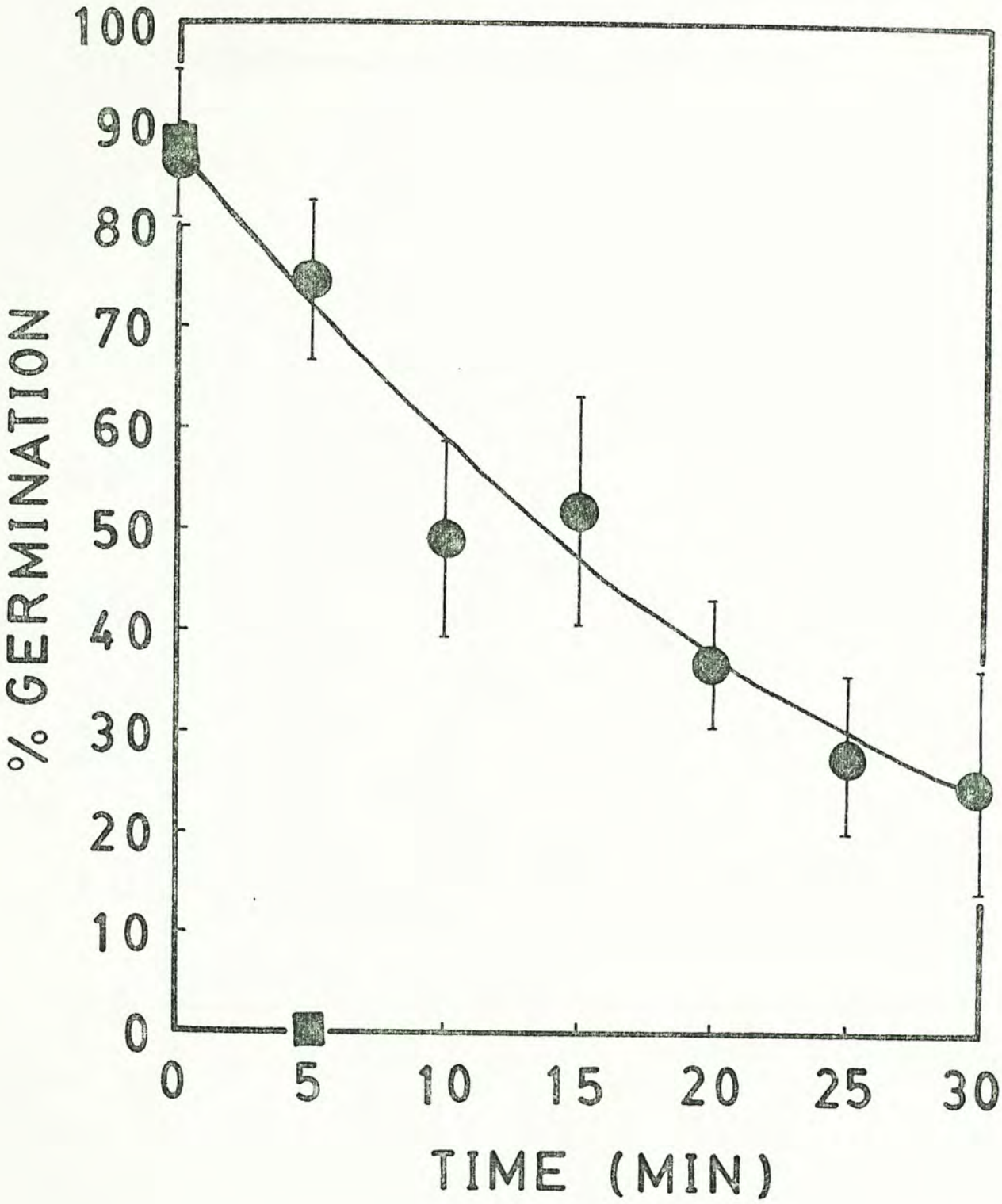


Fig. 2-7A : Resistancy of conidium to heat treatment. (●), 50 C; (■), 60 C.

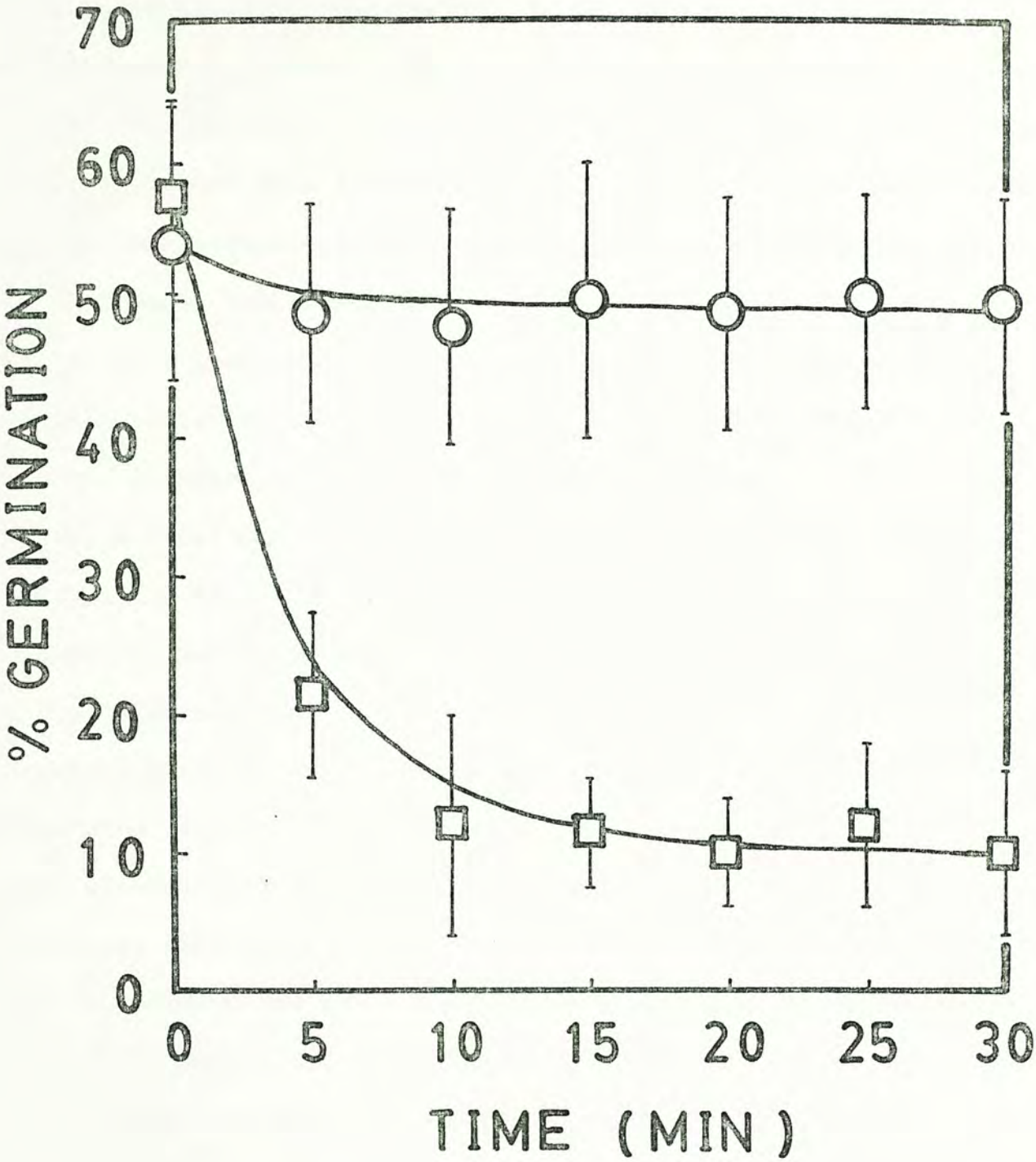


Fig. 2-7B : Resistancy of ascospore to heat treatment. (○), 50 C; (□), 60 C.

In this test, different carbon sources including monosaccharides, disaccharides, trisaccharide, polysaccharides and some derivatives were tested. The basic medium used was Czapek-Dox mineral nutrient(Table 2-1) solidified with 1 % Oxoid purified agar, and supplemented with different carbon sources. Basic medium without any supplements was used as control. Sterile distilled water and water agar(Table 2-1) were also used as comparing groups. The results were summarized in Table 2-2. Most of the carbohydrates tested stimulated the germination of both types of spores. Ribose, glucose, galactose, fructose, cellobiose, melibiose, raffinose, starch, m-inositol and mannitol were most stimulating to conidium germination, and salicin was inhibitory. Arabinose, ribose, glucose, cellobiose, maltose, raffinose and salicin were most stimulating to ascospore germination and galactose was inhibitory. The effects of different kinds of carbohydrates to conidium and ascospore germinations were not identical. Arabinose and salicin were used by ascospore but not effective or even inhibitory to conidium germination. Galactose, melibiose, trehalose, m-inositol and mannitol were effective to conidium but not used or even inhibitory to ascospore germination. Xylose, mannose, sorbose, sucrose, lactose, inulin and sorbitol were not used by both conidium and ascospore.

Malt extract(Oxoid), bacteriological peptone(Oxoid) and yeast extract(Difco) were the three common growth factors tested. The growth factors were added at three concentrations, 0.5, 1, and 2 % to the Czapek-Dox medium(Table 2-1) supplemented with 2 % glucose and solidified in 1 % agar. From the result tabulated in

Table 2-2 : Effect of carbon sources on the conidium and ascospore germination.

Carbon sources 2 %	% Germination +	
	Conidium	Ascospore
Monosaccharides		
D(+)Xylose	73±8	27±8
L(+)Arabinose	75±8	33±8 **
D(-)Ribose	86±8 **	39±10 **
D(+)Glucose	83±6 **	35±9 **
D(+)Galactose	85±6 **	20±8 *
D(+)Mannose	74±6	27±9
D(-)Fructose	83±7 **	27±8
L(-)Sorbose	76±8	22±7
L-Rhamnose	77±8	25±7
Disaccharides		
D(+)Cellobiose	83±6 **	30±7
Melibiose	85±5 **	28±8
Sucrose	76±9	28±9
D(+)Lactose	77±8	25±7
Maltose	70±8	30±7
Trehalose	80±7 **	28±7

Table 2-2 Continued

Trisaccharide				
Raffinose	81±8	**	31±8	**
Polysaccharides				
Starch	84±8	**	29±8	
Inulin	74±10		27±8	
Derivatives				
m-Inositol	81±6	**	28±8	
Sorbitol	77±8		29±8	
Mannitol	82±7	**	25±7	
Salicin	65±12	**	30±7	**
Control	73±7		25±6	
Water agar	47±9		10±4	
Sterile distilled water	0		0	

+ Using t-test, difference between control and the experimental groups was tested; *, significant; **, highly significant.

Table 2-3, all the three growth factors except 2 % malt extract were not effective to conidial germination but they were were much stimulating to ascospore germination. The differences among various concentrations were little, therefore 0.5 % of any of them would be satisfactory growth factors to both conidium and ascospore germinations.

Table 2-3 : Effect of malt extract, yeast extract and peptone on conidium and ascospore germinations.

Growth factors		% Germination	
		Conidium	Ascospore
Malt extract	0.5 %	84±6	63±8 **
	1 %	86±6	61±10 **
	2 %	88±5 **	60±8 **
Yeast extract	0.5 %	85±7	59±8 **
	1 %	85±7	58±9 **
	2 %	81±7	60±9 **
Peptone	0.5 %	86±7	59±10 **
	1 %	84±5	64±8 **
	2 %	84±7	63±10 **
Control+		83±6	35±9

+, Czapek-Dox medium enriched with 2 % glucose solidified in 1 % agar; **, highly significant.

Several metallic ions including sodium, potassium, calcium, magnesium and zinc were tested. All the mineral salts were added separatively at 0.5 mg per ml to the water agar medium enriched with 2 % glucose. The results were shown in Table 2-4, that zinc sulfate was inhibitory and all the others were germination stimulatants. The inhibitory effects of zinc to both ascospore and conidium were further investigated. Different concentrations of zinc ion varying from 0.1 to 10 µg per ml were slightly stimulating especially to ascospore germination. Over 50 µg per ml, zinc ion was inhibitory and it was more pronounced to ascospore(Fig. 2-8).

Table 2-4 : Effect of metallic ions on ascospore and conidium germination.

Metallic ions 0.5 mg/ml	% Germination			
	Conidium		Ascospore	
Sodium sulfate	87±8	**	57±8	**
Potassium chloride	84±8		49±9	*
Calcium chloride	86±6	**	47±7	
Magnesium sulfate	80±8		52±9	**
Zinc sulfate	51±9	**	12±7	**
Control+	80±7		43±8	

+, water agar enriched with 2 % glucose and solidified in 1 % Oxoid purified agar; *, significant; **, highly significant.

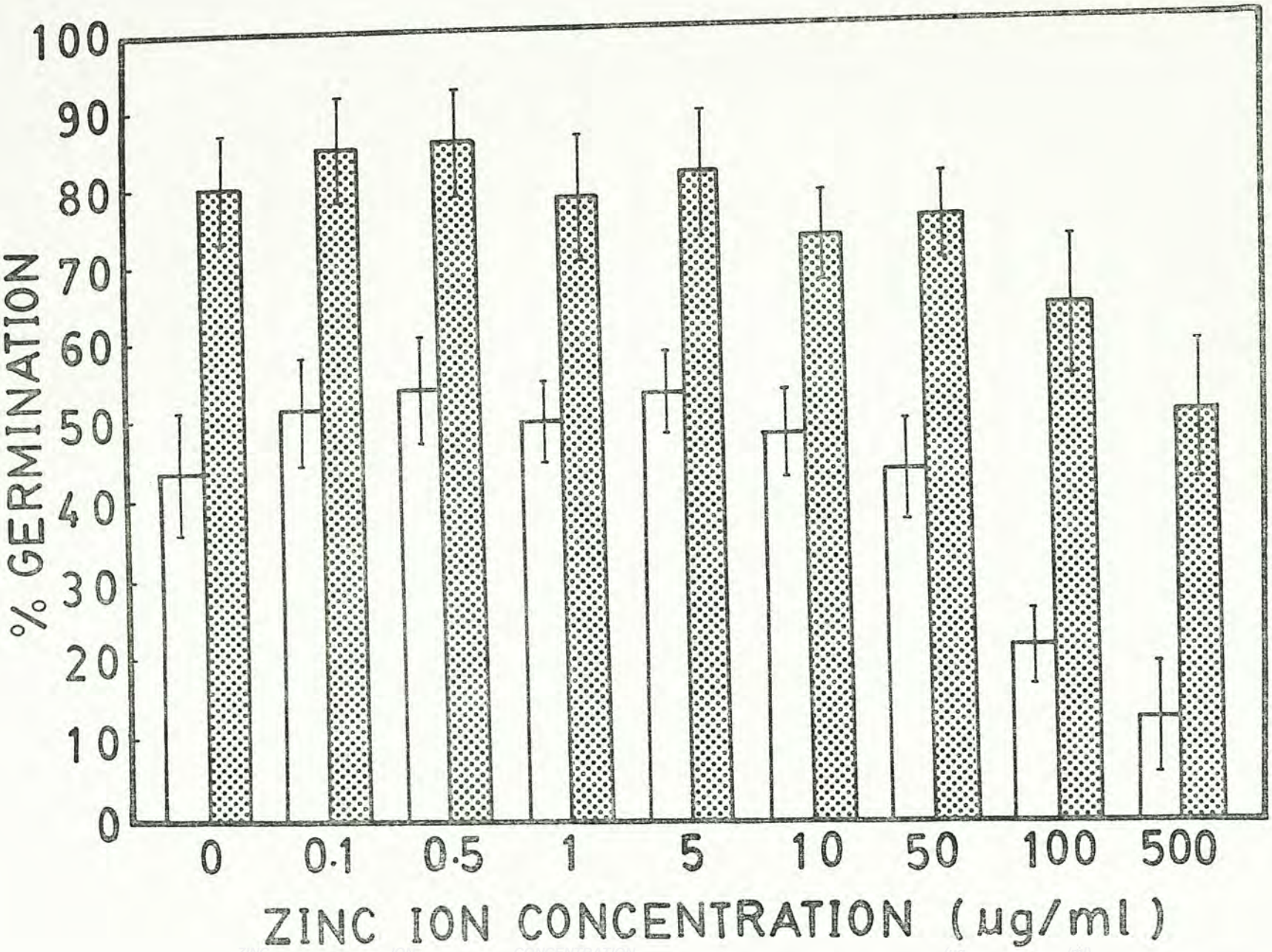


Fig. 2-8 : Effect of zinc ion on conidium(▩) and ascospore(□) germination.

Discussion

Germination is the protrusion of germ tubes from the spore and the whole process is generally divided into two stages, the stage before the appearance of germ tubes and the stage of germ tube formation(Schmit and Brody, 1976). Morphological and biochemical changes involved in such process have been investigated.

The swelling of spores before germ tube formation is a common phenomenon, especially in those air-borne fungal spores(Allen, 1965). The spores imbibe free water thus enlarge themselves and may be to several folds(Allen, 1965). The taking up of water is supposed not to be a result of metabolic activity, but the rehydration process can at the same time resume most physiological activities such as wall material synthesis and other reactions, that lead to the swelling of the spores(Anderson and Smith, 1972; Goldstein and Erb, 1972; Gottlieb and Tripathi, 1968; Martin and Nicolas, 1970). Still some others do not swell during the rehydration or taking up free water, like the chlamydospore of Phytophthora drechsleri(Cother and Griffin, 1974) and the ascospore of Chaetomium thermophile var. coprophile(Celerin and Fergus, 1971). The germination of both conidium and ascospore of Monascus purpureus did not show prominant swelling.

The ultrastructure of conidia is a popular subject and most of the reports for various fungi show quite similar architecture (Florance et al., 1972; Khan, 1975; Sargent and Payne, 1974). Most of the conidia have two layers as revealed by electron microscopy,

the outer electron dense and the inner electron transparent layers (Khan, 1975; Uduebo and Madelin, 1974) and some have minor layers (Florance et al., 1972). During the formation of germ tubes, new layer is generally produced linking to the inner layer and the germ tubes push out through the outer wall(Florance et al., 1972; Khan, 1975). The conidial germ tubes of M. purpureus as observed under light microscope also broke the outer wall and their wall were also continuous to the inner one. The ascospore of this species is comparatively small and no clear layers were detected. Together with other biochemical and biophysical studies, the mechanism of germination can be worked out(Benitez et al., 1976; Letourneau et al., 1976).

The number of germ tube produced by various fungi is different and even within the same species variation usually occurs. Usually one to two germ tubes are produced from one spore(Anderson and Smith, 1972; Celerin and Fergus, 1971; El-Buni and Lichtwardt, 1976; Paden, 1974). It was reported that in the conidium germination of Phytophthora parasitica and P. cinnamomi, up to 16 germ tubes per spore were produced. Most of the conidia of Monascus purpureus produced one germ tube and to a less extent two germ tubes were produced and in some of the larger conidia up to 6 germ tubes per conidium had been recorded(Plate 2-1). In ascospore germination of this fungus, only one and sometimes two germ tubes were produced. In the germination of ascospore of Chaetomium thermophile var. coprophile, globose vesicle is constantly formed preceding germ tube formation(Celerin and Fergus, 1971). Some of the ascospores

of Monascus purpureus produced one vesicle and followed by germ tube formation(Plate 2-2).

In addition to environmental factors, for example, pH, light, temperature, nutrient etc, the endogenous factors are of equal importance. Not all the fungal spores are ready to germinate shortly after their production, some require dormant period of weeks or months(Cochrane, 1958). The dormancy may be a result of immaturity, impermeability to gases and water(Cochrane, 1958) or the production of endogenous inhibitors(Gottlieb, 1973). This phenomenon is well represented by a number of aeciospore, uredospore, ascospore and some asexual spore such as conidium of various species. The breaking of dormancy is generally capable by heat treatment as in the ascospore of Neurospora spp. Others may be accelerated by chemical treatments, by low-temperature soaking, by mechanical abrasion or by variation in moisture(Cochrane, 1958). The latent period of conidium of Monascus purpureus was about one or one and half hours, so there was no dormant or resting period in such asexual spore. Though ascospore germination began at hour 2 from incubation and reached maximum percentage in hour 8, more than 40 % remained dormant that would probably require long term culture or special activations to germinate. An alternative is that they were dead spores.

The age of the spores also influences their germination. Ascospore of M. purpureus from day 5 slant culture exhibited maximum germination percentage. The reduced germination ability for spores from day 4 culture may be a consequence of immaturity while

spore from slants older than 5 days may show accumulation of endogenous inhibitors or pigments on the ascospore wall(Uduebo and Madelin, 1974). The conidia from day 10 culture also showed significant lower germination percentage. Such aging effect of conidia may also be attributed by the accumulation of pigments or endogenous inhibitors.

Diffusible germination inhibitors are generally postulated to explain the inhibitory effect of high spore density. Also, the germination of a number of fungal spores are not influenced by their densities(Celerin and Fergus, 1971). The stuation is completely different in the germination of ascospore and conidium of M. purpureus, whereas the germination percentage increased significantly with the increase of the spore density. It is supposed that some digestive enzymes secreted by some of the active spores degredated some macromolecules in the medium, possibly the disaccharides and the polysaccharides to form smaller molecules which in turn stimulated the germination of less active spores.

Moisture is not only important to spore germination , but also affects the viability and longevity of the spores. The atmospheric moisture is particularly required by the germination of some spores which is inhibited by free water, like the spores of Cercospora omphakodes(Judd and Peterson, 1972). The relation of relative humidity to the viability and longevity is rather complicated, some require extremely high, some intermediate, and still others require extremely low relative humidity(Celerin and Fergus, 1971; Cochrane, 1958; Dubin and English, 1975). As distilled water was

used to keep up the moisture instead of saturated sugar solution or mineral salt solutions generally used in relative humidity studies, the relative humidity within the closed chamber, the covered petri dishes, was believed to be near to saturation and it did give good germination. The drying up of the agar medium would stop the germination of both ascospore and conidium, and it is reasonable to believe that the free water was actually playing the most important role and the atmospheric moisture was only necessary in maintaining suitable amount of free water.

Atmosphere composition around culture also influences the germination of fungal spores. It has been demonstrated by many investigators that metabolic activities take place during the germination and uptake of oxygen(Cochrane, 1958; Fletcher and Morton, 1970; Gottlieb and Tripathi, 1968; Schmit and Brody, 1976). Beside oxygen, the amount of carbon dioxide in air also affects spore germination, whether inhibition as in conidium germination of Colletotrichum lindemuthianum(Arnold and Rahe, 1976) or stimulation as in the basidiospore germination of Polyporus dryophilus(Morton and French, 1974). The effect of atmosphere composition on spore germination of Monascus purpureus has not been investigated in this study, actually the culture chamber consisted just of atmospheric air.

Most of Monascus species are microthermophilic, growing in a temperature range of 18 to 45 C with optima at 30 to 38 C (Manandhar and Apinis, 1971), and the optimum temperature for the growth of M. purpureus is 30 to 35 C (Young, 1930). In this inves-

tigation, 35 C was the optimum temperature for both conidium and ascospore germinations. Though an optimum range occurred in conidial germination, still 35 C is choosen for further studies because it could be in cope with the ascospore optima.

The ascospore of M. purpureus is much more heat resistant than conidium. All the conidia were killed at 60 C only for five minutes. Incubation of the conidia at 50 C also rapidly minimized the germination percentage. On the other hand ascospore of this species is quite resistant to high temperature, whereas 50 C only slightly decreased the germination percentage. Though 60 C killed most ascospores, about 12 per cent still formed germ tubes after treating for 30 minutes. The high temperature resistancy of fungal spores highly depends on relative humidity, degree of spore hydration and the age of spores(Celerin and Fergus, 1971; Cochrane, 1958).

The hydrogen ion concentration, i.e. the pH of the medium is also one of the limiting factors in spore germination. Most fungal spores germinate best at pH 4.5 to 6.5 (Cochrane, 1958) and this optimum range is subjected to variations, actually the optimum pH for the germination of some spores varies with the medium composition(Callaghan, 1974) and temperature(Green et al., 1976). It also varies with the buffer system used in the investigation, as well demonstrated by the acetate and citrate-phosphate buffers used in ascospore germination study of M. purpureus. The optimum pH for conidium germination of this species was rather typical with a narrow range of optimum. Contrary, the optimum value for ascospore germination was much wider ranging from 2.6, the lowest value tested,

to 6 and it skewed to acidity.

Some fungal spores already have enough stored energy for germination, and lipids have been shown to be popular fuel reserved(Sargent and Payne, 1974), so these spores usually require no exogenous carbon sources and will germinate just in distilled water(Sargent and Payne, 1974), or simply on filter paper(Verma and Petrie, 1975). There are also numerous fungal spores which germinate only in untrient media containing complete or special constituents. According to Cochrane, the nutrient requirements can be divided into three types : complete independence, partial dependence and complete dependence(Cochrane, 1958). Since both conidium and ascospore of M. purpureus could not germinate in distilled water, so it is classified as a completely nutrient dependent species.

Nearly half of the carbohydrates tested were effective to both conidium and ascospore germination and most of these carbohydrates were stimulating. The result shown in Table 2-2 reflects the different exogenous enzyme patterns secreted by these spores. As a whole conidia could utilize larger molecules like starch, m-inositol, mannitol etc. Enzymes of conidia may be descended directly from the vegetative hyphae, for example the amylase. Amylase is produced by vegetative hyphae therefore the mold is called "starch fungus" and there was evidence of its existence in conidia but not in ascospore. Because of the extremely high germination rate of conidia, the stimulating effect of growth factors like malt extract, yeast extract and peptone were not significant, but these growth factors were most favorable for ascospore germination. This

result shows that conidia may contain enough vitamins, amino acids, mineral salts and other growth factors but carbon sources.

The influence of metallic ions on conidium and ascospore germination also reveals that conidia contain almost enough essential ions, since the metallic ions tested slightly increased the germination percentage, for example sodium and calcium. But potassium and magnesium were insignificant. The stimulation of ascospore germination by metallic ions was more prominant, sodium, potassium and magnesium were all stimulating. Zinc at 0.5 mg per ml was extremely inhibitory to both ascospore and conidium germination and it was more harmful to ascospore. It has been well investigated that zinc is essential for fungal growth, becoming toxic only at high concentration(Ross, 1975). Reported by McHan and Johnson, zinc is a trace element that greatly increase the growth of this fungus and the optimum concentration is 800 µg per liter, and it is supposed to be related to the regulatory mechanism of carbohydrate and nitrogen metabolism(McHan and Johnson, 1970). Zinc ion concentrations from 0.1 to 10 µg per ml was slightly stimulating to the germination of these spores especially to ascospore. Another type of zinc effect on fungal growth is the study of the dimorphism of Candida albicans by Yamaguchi. He found that zinc completely reversed the filamentous phase of growth and concentration above 10 µM was most effective (Yamaguchi, 1975).

As a conclusion, the germinations of conidium and ascospore are quite different. Besides the morphological differences, all the data show that they also differ physiologically in several as-

pects, like pH response, high temperature resistancy, carbon source utilization, metallic ions and growth factors effects etc. Conidium contains nearly enough essentials for germination as compared to ascospore and it is more close to vegetative hyphae.

CHAPTER THREE

EFFECTS OF FAST-NEUTRON, RUBY LASER AND SOFT X-RAY ON CONIDIUM AND ASCOSPORE GERMINATION OF *MONASCUS PURPUREUS* WENT

Introduction

Ionizing radiations, causing the formation of ion pairs in the energy-absorbing matters, include electromagnetic radiations such as γ-ray and X-ray, and particulate radiations such as β-ray, α-ray, proton and neutron, which differ in charge and mass. Though the initial processes of electromagnetic and particulate radiations are different, their effects are similar. The electromagnetic radiations can activate electrons to be released. Particulate radiations bombark on molecules to release electrons or protons which in turn can cause ionization directly or indirectly, i.e. the final effect of the radiations on molecules is ionization (Bacq and Alexander, 1966).

The effects of radiation at molecular level, cellular level and organismic level have been investigated and reviewed (Bacq and Alexander, 1966; Nygaard et al., 1975). But the responses of different living things to different kinds of radiation vary greatly that generalization is usually difficult. Biologically ionizing radiations are employed in inducing mutation and it is more convenient than using mutagenous chemicals. They are also currently used for medical purposes, sterilization and preservation in food indus-

try(Nygaard et al., 1975).

The introduction of laser in biological research is rather new. Basically, laser is only electromagnetic wave of visible wavelength around 650 nm, but with extremely high intensity. Generally it causes burning to the irradiated parts but not ionization.

The present study was to show the effects of X-ray, fast-neutron and laser on the spore germinations of Monascus purpureus and the influence of a redox dye, toluidine blue, on the spores after irradiations.

Materials and Methods

A. Irradiation of X-ray

The spores were collected and inoculated to malt extract agar blocks on micro-culture slide in the special petri dish sets as described in Chapter two. Shortly after the inoculation the sets were placed under the X-ray tube with the covers removed, in dim red light and at room temperature. A Picker soft X-ray generator was used throughout the study. Voltage of 65 KV and current in 5 ma were set and the effects of different exposure time were investigated. After the radiation treatment, the spore cultures were incubated at 35 C in dark to germinate. The spore germination was observed under light microscope and counted at 200x magnification.

B. Irradiation of fast-neutron

The micro-culture slides with spores on agar blocks were

taped on a plank and placed at 20 and 40 cm from the fast-neutron source as shown in Fig. 3-1. Energy of the neutron generator was 14 MeV producing 5 x 10^9 neutrons per cm^2 per sec. Exposure time of 2 h 20 min, 3 h, and 3 h 20 min were used.

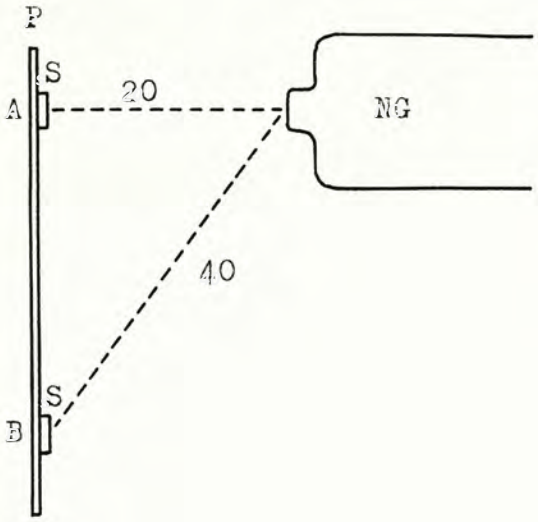


Fig. 3-1 : Irradiation of spores by fast-neutron. Spore cultures(S) were taped on a wooden plank(P) at 20 cm(A) and 40 cm(B) from neutron source of the generator(NG).

C. Irradiation of laser

A ruby laser was used in this test. One to two pulses of 1 msec per pulse were used and a total energy up to 35 J were studied. The wavelength of the laser light was 694.3 nm.

Results

A. Effects of X-ray, fast-neutron and laser radiations

As measured by a radiameter, the radiation emited by

X-ray generator was extremely low, i.e. about 0.1 r per hr. As shown in Table 3-1 and 3-2, the germination of both ascospore and conidium was not affected.

Table 3-1 : Effect of X-ray on the germination of conidium

Radiation 0.1 r/h	% Germination		
	0	0.01 %*	0.002 %*
0 min	93±4	96±4	93±6
1 min	93±4	92±5	91±5
3 min	90±6	93±5	93±6
5 min	91±6	94±5	93±5
10 min	92±5	95±4	95±4

* One loopful of 0.01 % or 0.002 % toluidine blue was added to each spore culture.

Table 3-2 : Effect of X-ray on the germination of ascospore

Radiation 0.1 r/h	% Germination		
	0	0.01 %*	0.002 %*
0 min	57±6	57±7	61±5
1 min	61±6	63±5	66±5
3 min	59±7	61±7	66±6

5 min	58±6	65±4	64±4
10 min	60±5	62±6	59±5

* One loopful of 0.01 % or 0.002 % toluidine blue was added to each spore culture.

On the other hand, irradiation of fast-neutron for three hours was fatal to both conidia and ascospores. The conidia placed at 20 cm from the neutron source showed a greatly lowering germination percentage of 10 %, while those placed at 40 cm only as low as 58 %. Exposure for 3 h and 20 min killed all the conidia (Table 3-3). Ascospores could resist longer fast-neutron radiation. About 13 and 16 % of ascospore germination were still observed after treatment for 3 h 20 min placed at 20 and 40 cm away respectively. Irradiation of ascospore for 2 h 20 min or less was not harmful to the ascospore germination. So the response of conidia to fast-neutron radiation was rather even while that of ascospore was less sharp.

Though the agar medium was scorched by the laser radiation for one to two pulses, the germination of both conidium and ascospore were not affected and the values were not significantly different from the control.

B. Influence of toluidine blue on the radiation effects

Toluidine blue was dissolved in sterilized distill water and diluted to 0.01 % and 0.002 %(w/v). One loopful of this dye was spread through the surface of the agar blocks. Then, one loopful

Table 3-3 : Effect of fast-neutron on the germination of conidia

Neutron radiation	Distance from neutron source	% Germination		
		0	0.01 %*	0.002 %*
0		94±4	91±5	95±3
3 h	40 cm	58±9	55±10	56±10
	20 cm	10±6	24±7	32±9
3 h 20 min	40 cm	0	0	0
	20 cm	0	0	0

* One loopful of 0.01 % or 0.002 % toluidine blue was added to each spore culture.

Table 3-4 : Effect of fast-neutron on the germination of ascospore

Neutron radiation	Distance from neutron source	% Germination		
		0	0.01 %*	0.002 %*
0		58±7	57±8	61±6
2 h 20 min	40 cm	61±5	60±9	62±7
	20 cm	58±5	58±8	57±6
3 h	40 cm	23±5	42±7	43±8
	20 cm	19±6	19±4	21±5

3 h 20 min	40 cm	16±6	23±6	20±6
	20 cm	13±4	16±4	17±6

* One loopful of 0.01 % or 0.002 % toluidine blue was added to each spore culture.

Table 3-5 : Effect of laser on the germination of conidia and ascospore

Laser radiation(J)	% Germination	
	Conidium	Ascospore
0	95±4	54±12
6.9	96±3	54±10
13.8	97±3	52±12
17.5	96±2	49±12
35	95±5	53±9

of spore suspension was introduced on them. After radiation treatments and then germination determination, this dye was shown to have some protecting effect. It was prominant in case of fast-neutron treatment. In X-ray radiation study, the spores were not killed at all, so there was no protection phenomenum observed. With the presence of toluidine blue, more than double or triple amount of conidial germination were resumed after fast-neutron radiation treatment for 3 h at 20 cm from the neutron source (Table 3-3). The ascospores were also protected, more apparent and significant at 3 h radiation and 40 cm from the neutron source. The germination was doubled in the presence of toluidine blue. The dye used at these concentrations could only resume part of the spore germinations as compared to the nonirradiated control. Also, such protection was likely to be restricted to certain radiation dosages which were not quite similar for conidia and ascospore, 3 h at 20 cm for conidia and 3 h at 40 cm for ascospores.

Discussion

Effects of various radiations on fungal spore germination have been studied, namely ultraviolet light (Curtis, 1970; Janitor, 1975), γ-rays (Scott et al., 1972), X-ray (Klein and Klein, 1962), fast- neutron (Hamann, 1971), laser (Macmillan et al., 1966), and visible lights (Chang et al., 1974). The ionizing radiations are usually employed in inducing special mutants in genetic studies (Malling and DeSerres, 1973; Webber and DeSerres, 1965).

The action of ionizing radiations on biological systems can be divided into several levels: molecular, cellular, and organismic level. Though the mechanisms of ionizing of different radiations are dislike, ion pairs can be produced. Macromolecules could be denatured by breaking of chains, crosslinking of chains, or changing the steric configuration under ionizing radiations. On cellular level, radiation may kill cells, destruct subcellular organelles or delay cell division. The whole organism may be killed, somatic harmed or genetic changed(Bacq and Alexander, 1966).

The studies of radiations on fungal spores generally fall into two groups: effects on germination and survival percentages, and mutation which will be discussed in the following chapter.

X-ray and Y-ray are similar radiations and cause death of fungal spores only at high dosage of kiloroentgens(Klein and Klein, 1962; Scott et al., 1972). Low dosage from soft X-ray generator is usually not discussed. Actually, as shown in Table 3-1 and 3-2, irradiation for even 10 min by soft X-ray could not affect the germination of conidia and ascospores in M. purpureus.

The fast-neutron used was rather energetic as compared to X-ray, irradiation with 2 h 20 min and above killed effectively the spores, and ascospore as a group in particular was more resistant. The resistancy of conidia and ascospores may be due to : (1) thick wall, and (2) protectants. Since ascospores of M. purpureus are pink colored, the pigments might act as radiation protectants. The protection properties of pigments have already been reported. Carotenoids, the most popular investigated pigments,

exist in bacteria and fungi(Valadon, 1976). These pigments protect the bacterium Sarcina lutea Schroeter against lethal laser radiations (Macmillan et al., 1966). Though the survival curves of pigmented and pigment-inhibited strains of Halobacterium cutirubrum (Lochhead) Elazari-Volcani after UV irradiation are similar, the photo-reactivation effieiency is much higher in pigmented strains(Hescox and Carlberg, 1972). The black-brown colored Aspergillus carbonarius (Bainier) Thom. conidia are more resistant to UV radiation than colorless mutants(Curtis, 1970). Other pigment also known as radiation protectant is melanin which is a black one(Ellis and Griffiths, 1975).

In nature, laser is not an ionizing radiation, but due to its high intensity it can cause damages even down to subcellular level, like chromosome abnormalities(Robertson and Wilson, 1970). In case of laser radiation, some redox dyes such as toluidine blue which has absorption maxima concording to the wavelength of laser light, can act as exogenous photoreceptors transferring the absorbed energy to the living systems, thus killing of organisms in this way is an indirect process. Without such exogenous receptors, most of the bacteria are not killed and Saccharomyces cerevisiae Meyen ex Hansen is not affected at all even in the presence of dyes, by a continuous-wave gas laser irradiation (Macmillan et al., 1966). The fungus Aspergillus niger van Tiegh. has also been shown to be undamaged by ruby laser irradiation up to 76 J (Klein et al., 1965). The conidia and ascospores of M. purpureus were also shown to be unaffected up to 35 J.

According to Bacq and Alexander, the protection of protective agents can occur in two ways : by energy transfer and by repair of an unstable intermediate. Aromatic compounds are good energy traps(Bacq and Alexander, 1966). Since toluidine blue is also an aromatic compound, therefore it could contribute its protective properties. Dosage of radiation higher or lower than certain amount still caused damages. It is supposed that the toluidine blue be activated by the fast-neutron energy higher than certain level and can interact with some free radicals which are also produced by radiation and responsible for the killing of cells. When energy is lower than certain level, there will be no toluidine blue activation. As the energy is higher than certain level, no protection will be expected since overwhelming quantity of action free radicals are produced.

CHAPTER FOUR

MORPHOLOGY OF FAST-NEUTRON, RUBY LASER AND SOFT X-RAY INDUCED STRAINS OF *MONASCUS PURPUREUS* WENT

Introduction

The induction of mutants is sometimes not only valuable in mycological investigation, but also with extensive practical interests. Those mutants with point mutation on the chromosomal nucleic acid usually act as gene markers and they are used in gene crossing studies in order to reveal the genetic pattern of these organisms. *Neurospora* species have been well known in this aspect (Tan and Ho, 1970). In the identification of some pathogenic races of some species, mutants are valuable as markers, and it is more convenient in manipulation than by physiological comparison, for example, mutants of *Phytophthora infestans* (Mont.) deBary (Shattock and Shaw, 1975). In industrial practice, special mutants are induced to produce some required products more efficiently, for example the production of antibiotics (Lemke and Nash, 1972). In physiological studies, mutants provide many helpful materials that will certainly facilitate the studies of some features, such as synthetic pathways, morphogenesis, etc.

Some strains of *Monascus* species were selected to produce much higher glucoamylase activity, which would be more promising in glucose manufacturing industry (Ho et al., 1973). Since the

pigments synthesized by Monascus spp. can be used as natural coloring materials, the induction of mutants of high pigmentation will be valuable in food industry. Actually, one highly pigmented mutant was isolated (Su et al., 1973). But no further studies on these mutants have been reported.

The artificial induction of mutants is carried out by the using of mutagens, among which chemicals such as N-methyl-N'-nitro-N-nitrosoguanidine (Lemke and Nash, 1972) and ionizing radiations such as X-ray, γ-ray, and UV etc. are most familiar ones. In practice, ionizing radiations are more convenient to manipulate.

In this study, strains were induced by fast-neutron, ruby laser and soft X-ray irradiation and the macro- and micromorphology of these strains were also investigated.

Materials and Methods

A. Selection of strains

After the irradiation treatments, two methods were used to obtain single spore cultures. The first one was simply picking up the germinated spores from the agar blocks with a platinum needle and transferred them to malt extract agar slants. The second one was the modified dilution plating method. Drops of sterile distilled water were laid on the agar blocks and the spores were disloged into the water with the help of a platinum needle. The droplets containing spores were then pipetted to agar plates and spread by a bended glass rod. After incubated at 35 C for 2 days, fungal

colonies were observed and each colony was then subcultured separately. Since the conidium has one or more nuclei and ascospore contains one or two in each spore, complementary reactions would probably mask the appearance of gene alterations. So the single spore cultures were transferred for several times. Sectors of different macromorphology were then isolated and cultured to obtain pure strains. Generally, the pigmentation, growth rates and morphology were adopted as selecting criteria.

B. Comparison of growth rates

The radial growth on agar plate and dry weights attained in liquid medium culture of the strains were compared.

With an inoculation needle, the strains were transferred to petri dishes containing 25 ml malt extract agar medium and incubated at 35 C in darkness for 10 days. The diameters of fungal colonies were measured. A total of 10 colonies for each strain were determined.

Mycelium discs of the strains were cut with a cork borer of 0.6 cm diameter from the margin of 10-day cultures. Each disc was cultured in 50 ml malt extract liquid medium in 250 ml conical flask. The 5-day cultures were filtered through pre-weighed Whatman No. 1 filter paper and the mycelia were dried to constant weights in oven at 80 C for about 12 to 16 hours. The final pH values of cultural medium were also determined. The values were the means of every 10 determinations.

C. Production of conidium and ascospore

The production of conidia and ascospores by the strains

induced were also compared. Different strains were inoculated to the center of agar plate and cultured for 10 days. Each colony was scalpelled and blended in a Mitsubishi blender with 25 ml distilled water at high speed for 30 sec. The effluent was filtered through single layered cheesecloth and the spore density was determined with a haemacytometer. Total numbers of conidia and ascospores from each colony were then counted. The values were the means of every 10 determinations.

D. Macro- and micromorphology study

The development of color and the external morphology of these strains on malt extract agar medium cultured at 35 C in total darkness were noted and their color photographs were also made.

Micromorphology of the strains was observed under light microscope. The main features studies were hyphae, crozier, cleistothecia, ascospores, conidia and pigment crystals. Acid fuchsin was used as a stain especially for the examination of sexual reproductive structures and conidia. Some special features were shown by photography.

Results

A. Strains obtained

Hundreds of spores were isolated from each irradiation treatment. After repeated subculturing and purification, three strains from fast-neutron, four from X-ray and one from laser irradiation ions were selected. All of these new strains came from asexual conidium. Therefore mutants could be primary(P) in first isolation

or secondary (S) after subculturing. Strains N4S, N11S and N14S were isolated from fast-neutron treatment. X2P, X3S, X4P and X5S were produced by X-ray irradiation, and L2S was isolated after laser irradiation. These strains were quite stable and no back mutation to the wild-type had been observed during this study.

B. Growth rates of the strains

Growth rates of these strains were variable. The dry weights as well as the radial growth were compared. Among these strains, N14S, X3S, X4P and X5S had higher growth rates. They grew 50 % or more faster than the wild-type. Growth rates of strains N4S, N11S, X2P and L2S were more or less the same as wild-type (Fig. 4-1).

The final pH values of liquid cultural media also reflected the growth conditions of these strains. Those of fast growing strains, such as N14S, X3S, X4P and X5S had final pH values higher than 4. Growth of N14S was the fastest and the final pH of its cultural medium was also the highest, 5.1 (Table 4-1).

C. Macromorphology

Strain N14S was practically albino (Plate 4-4A). Strain X4P was tinged with some red color but spread not evently (Plate 4-7A). Red pigment also deposited on the center of X3S culture, but to much lesser extent than the wild-type (Plate 4-6A). Pigmentation appeared on day 2 and became deeper as the cultures grew older. The 2- and 3-day cultures of N4S, N11S, X2P, L2S and wild-type were orange colored. The 7-day cultures of N11S and L2S still remained orange color, while others became red colored. At day 10 all these cultures became deeply colored than the wild-type. The surface of 10-day culture of N4S was somewhat grey colored. The

Table 4-1 : Final pH of the liquid medium.

Strains	Final pH *
Wild-type	3.6
N4S	3.7
N11S	3.3
N14S	5.1
X2P	3.5
X3S	4.4
X4P	4.1
X5S	4.1
L2S	3.5

*, Initial pH of the medium before autoclaving was 5.5.

X5S culture was slightly grey colored on day 2 and day 3. Afterwards, red pigment was deposited, more on underneath of the mycelium (Plate 4-8A)

The undulating surface of wild-type culture on malt extract agar appeared to be radiating with ridges and grooves. This radiating appearance also exhibited by all other induced strains. The radiating appearances of slightly pigmented strains, X3S and X4P, and the albino strain N14S were the results of intercrossing and radiating bundles of hyphae. Much lesser radiating grooves and

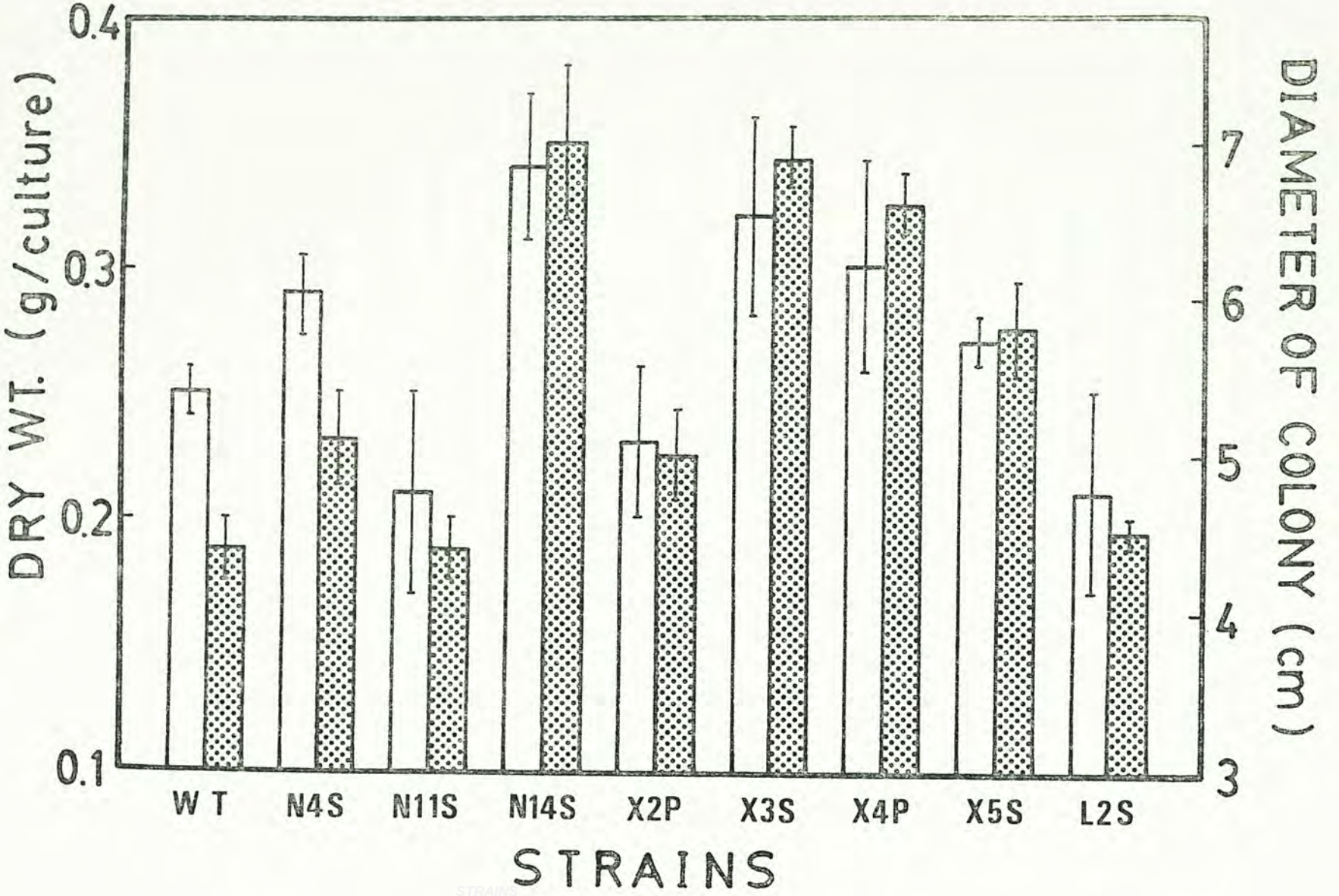


Fig. 4-1 : Growth of wild-type(WT) and different strains on solid and liquid medium. (□), dry weights; (▩), diameter of colony.

ridges were exhibited by N4S and X5S. It was even fewer in N11S and L2S.

The surfaces of N4S, N11S, X2P, X4P, L2S and wild-type were not furry. Aerial hyphae were observed on the surfaces of N14S, X3S and X5S cultures, so that these strains appeared to be cottony and furry, masking the radiating ridges on their mycelia. Some deep red to brown colored hyphae erected on the center of N4S colony, but the number of these hyphae were comparatively limited and it did not give a furry appearance. These aerial hyphae beared chains of conidia. Mycelium pads of 7-day cultures of N11S and L2S were bulging, and they became flat on day 10, while others remained flat during the whole period of 10 days except X2P.

The external morphology of strain X2P was the most peculiar one. The colony was red colored as the wild-type. The radiating ridges and grooves were more delicate and contorted. Mycelium pad of this strain was elevated with the agar medium lifted up from the bottom of the petri dish. The center of this colony usually ruptured to form apertures and the hyphae consequently grew to the back side of agar medium (Plate 4-5A).

D. Micromorphology

The mycelia of wild-type, N4S, N11S, X4S and L2S were fragile and the hyphae could be easily broken with a needle or cork borer. Those slightly pigmented strains X3S, X4P and the albino N14S were more sturdy. So it was not easy to cut mycelium disc from colonies of these strains.

As revealed by light microscope examination, N14S, X3S and

X4P had comparatively thicker hyphae with fewer branching. Pigmented hyphae could only be found occasionally in these strains. Hyphae from wild-type and other strains were pigmented and narrower. Pigments of these colored strains especially N4S, N11S and L2S were dense. Besides heavily pigmentation of the hyphae of these three strains, red and yellow crystals were also found surrounding the hyphae and the crystals sometimes aggregated into large particles outside the hyphae.

Young hyphae from the margin of X2P colony were filamentous and pigmented (Plate 4-5C). But mycelium of this strain from other part of the colony composed of two types of hyphae : the filamentous hyphae composed of elongated cells and the abnormal type composed of isodiametric cells (Plate 4-5 F and G). The latter type generally out-numbered the filamentous hyphae in older mycelium. Exceptionally excess number of oil droplets were found within the isodiametric cells (Plate 4-5 F).

Reproduction of most strains completely reversed the general mode happenning in wild-type. Instead of producing mainly ascospore by 4-day or older culture in wild-type on malt extract agar , most strains produced conidia. Though sexual reproduction of X5S was occasionally observed, it was seldomly found any ascospore in the effluent of its colony during the spore density determination. The only exceptional case was X4P which produced nearly equal amount of conidia and ascospores (Table 4-2). From 10-day wild-type cultures, cleistothecia usually contained a number of ascospores. Lesser ascospores were found in each mature cleistothecium of X4P

Table 4-2 : Production of conidia and ascospores in wild-type and various strains.

Strains	Spores/colony($x10^5$)	
	Conidium	Ascospore
Wild-type	6.4± 3.0	93.0±30.0
N4S	98.0±13.0	0
N11S	0.8± 0.8	0
N14S	1.6± 0.7	0
X2P	2.5± 0.6	11.0± 3.0
X3S	55.0±14.0	0
X4P	1.8± 0.4	1.2± 0.2
X5S	150.0±30.0	Trace
L2S	0.5± 0.4	0

and X5S and correspondently the size of cleistothecia was also smaller (Plate 4-5, 4-7 and 4-8).

The formation of crozier in ascomycetes is generally followed by the differentiation of ascocarp. Crozier could be found in X2P, X3S, X4P, N11S, L2S and wild-type. Mature cleistothecia containing ascospores were not detected from X3S, N11S and L2S. Though fertile cleistothecia were produced by X5S, crozier had not yet been observed. However, ascogenous hyphae and stages in cleistothecia differentiation were observed. Cleistothecium-like structures were

found in cultures of X3S, N11S and L2S. These structures were believed to be sterile or protocleistothecia (Plate 4-3, 4-6 and 4-9).

The ontogeny of ascus and ascospore of this species is still confused. Single ascus was differentiated from the cleistothecium of X2P leaving large epiplasm (Plate 4-5D).

Discussion

Table 4-3 summarizes the characteristics of various strains studied. The vegetative hyphae as well as the spore productions of these strains were different from the wild-type. Protoperithecial production in Sordaria brevicollis Olive et Fantini and Neurospora crassa Shear et Dodge were shown to be controlled by genes (Bond and MacDonald, 1976; Tan and Ho, 1970). The laser, X-ray and fast-neutron induced strains of Monascus purpureus had various types of spores production and similar changes in genes were expected.

The growth rates of the strains seem to be related to the pigment production. The texture of the hyphae is also affected. The pigments produced by these strains may probably inhibit the synthesis of cell wall constituents, such as glucans, cellulose, chitin, etc., thus reduce the growth rates and render the hyphae more fragile. The synthesis of pigments may also be a competiting process to the synthesis of cell wall materials.

The effects of pigment production to the morphogenesis have been investigated. A purple pigment isolated from Panus tigrinus (Bull ex Fr.) Sing enhanced fruiting and it was supposed to be precusor in the fruiting pathway or by-product which could affect

Table 4-3 : Characteristics of wild-type and different strains on malt extract agar plate.

Strains	Pigments	Mycelium *			Reproduction			
		Aerial hyphae	Isodiametric cells	Texture	Conidia	Crozier	Protocleis-tothecia	Cleistothecia with ascospores
Wild-type	++++	-	-	F	++	+	+	++++
N4S	++++	+	-	F	+++	-	-	-
N11S	++++	-	-	F	+	+++	+++	-
N14S	-	++	-	S	+	-	-	-
X2P	+++	-	++	F	+	+	+	++
X3S	+	++	-	S	+++	+	+	-
X4P	Trace	-	-	S	+	+	+	+
X5S	++	++	-	F	++++	-	+	Trace
L2S	++++	-	-	F	+	+++	+++	-

*, All strains have filamentous hyphae.

+, Present; -, absent; number of + shows the abundancy; F, fragile; S, sturdy.

the fruiting process (Faro, 1972). Mutant induced by Su et al. also had high pigmentation, large amount of conidia and a few ascospores. They supposed that the ascospore differentiation is blocked after UV irradiation and conidia are thus differentiated in a extensively enhanced scales as a alternative means of reproduction. They also showed that pigments were accumulated within conidia. They proposed a mechanism that the excessive production of pigments is a result of inhibited ascospore production and stimulated conidia formation (Su et al., 1973). In strains X3S and X5S, conidia production was extensively enhanced, but the pigment production was much reduced. Strains N11S and L2S synthesized double amount of pigment, but only a few conidia were produced. Strain X4P could differentiate into a small number of conidia and ascospore, the mycelium was only slightly colored. These results do not agree with Su et al.'s explanation, only N4S agreed with their trial. Ascospores were slightly pink colored and conidia were somewhat hyaline and colorless in wild-type and all the strains studied.

Sexual reproduction of different organisms can be stimulated by various means, chemical or physical treatments; for example, sexual hormones in Dictyostelium purpureum Olive (Lewis and O'Day, 1976), magnesium and calcium ions in Phytophthora fragariae Hickm. (Maas, 1976), manganese ion in Aspergillus nidulans (Eid.) Wint. (Zonneveld, 1975A and B), polyols in Neurospora tetrasperma Shear et Dodge (Viswanath-Reddy and Turian, 1975), light in Leptosphaerulina briosiana (Pollacci) Graham et Luttrell (Moyer and Leath, 1976), and temperature in Hypomyces solani (Mart.) App. & Wr.

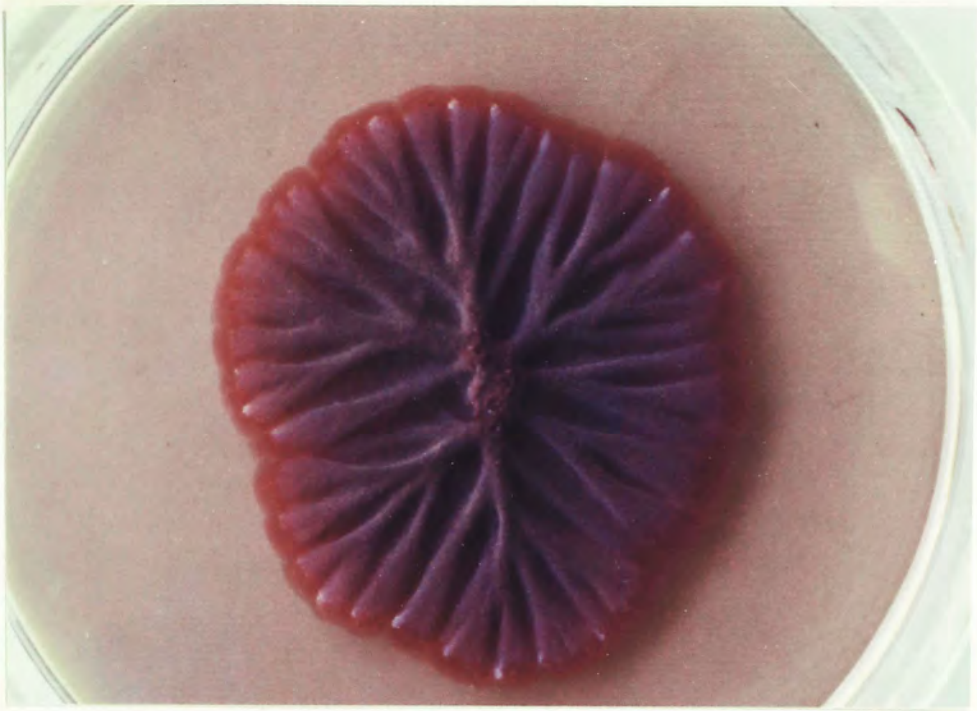
f. sp. cucurbitae Snyd. & Hans (Wilson and Baker, 1969) and Mucor miehei Coon. et Emers.(Lasure and Ingle, 1976). Asexual spore production of some species is also stimulated by some factors, for example, an unknown compound extracted from soil pseudomonads in Phytophthora cinnamomi Rands (Ayers, 1971) and light in Botrytis cinerea Pers.(Tan, 1975A and B). These factors proved to be active to stimulate spore formation in other species may also be effective to resume the spore production especially the inhibited ascospore formation of these strains. Since different stages in ascospore formation could be found in these strains, further study on the difference between these strains and the wild-type could facilitate the understanding of ascospore differentiation. Strain X5S also provides material for conidiation study.

Interrelation among pigmentation, conidiation, ascospore formation and growth of Monascus purpureus may be complicated. They may be regulated by a number of free and related genes.

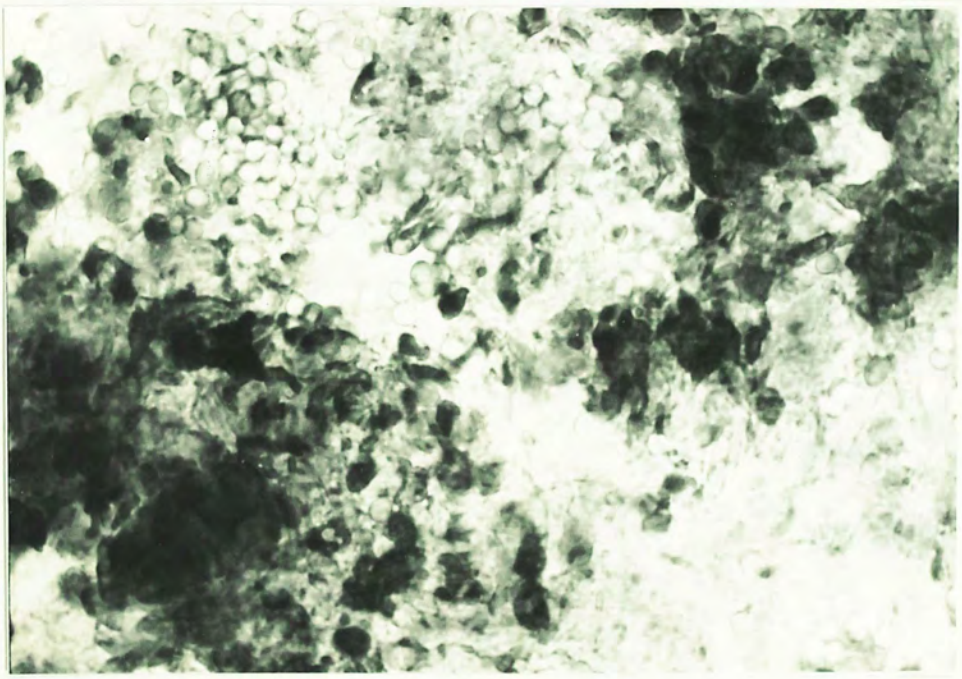
All the strains came from conidia and it may be due to : (A) higher resistancy of ascospore to ionizing radiations as discussed in Chapter Three, (B) low germination percentage and thus less single ascospore cultures succeeded.

Strains N11S and L2S were identical in all the characters examined, so similar mutation would have taken place following the irradiation of fast-neutron and ruby laser.

Further studies on the photoresponses, pigmentation and antibiotic activity of these strains will be mentioned in Chapter Five and Six.



A



B

Plate 4-1 : Morphology of wild-type.

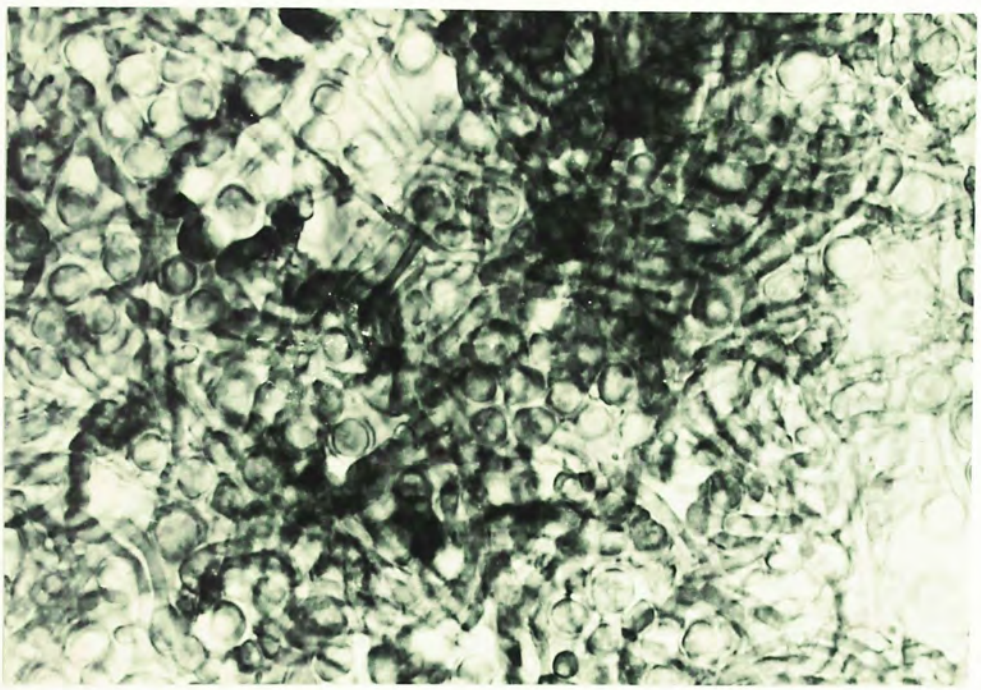
A. 10-day culture of wild-type, surface view. x3/2.

B. Mycelium from 10-day culture showing numerous cleistothecia with ascospores. x400.

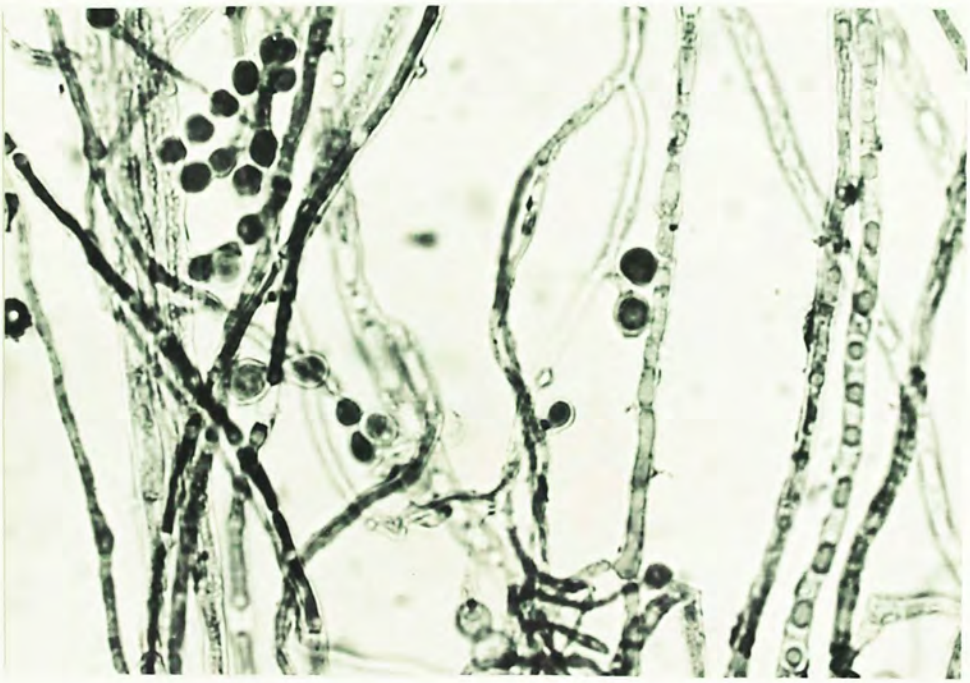
A

Plate 4-2 : Morphology of strain M4S.

A. 10-day culture with slightly grey surface and brown aerial hyphae on the center of the colony. Left, surface view; right, underneath view. x 2/3.



B

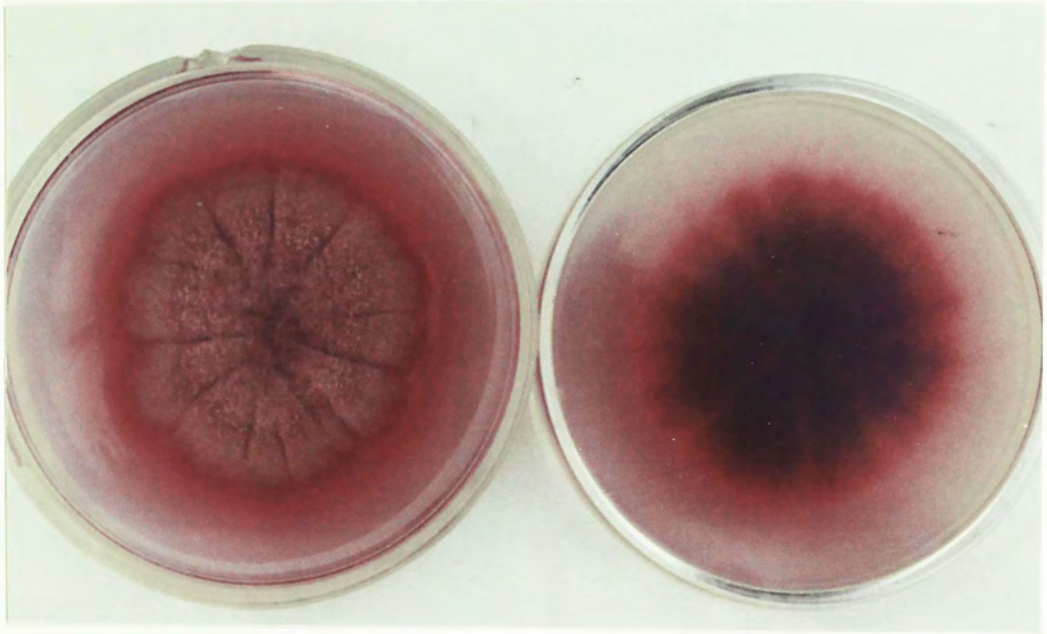


C

Plate 4-2 (Continued)

B. Mycelium with numerous conidia, but no sexual reproduction. x 400.

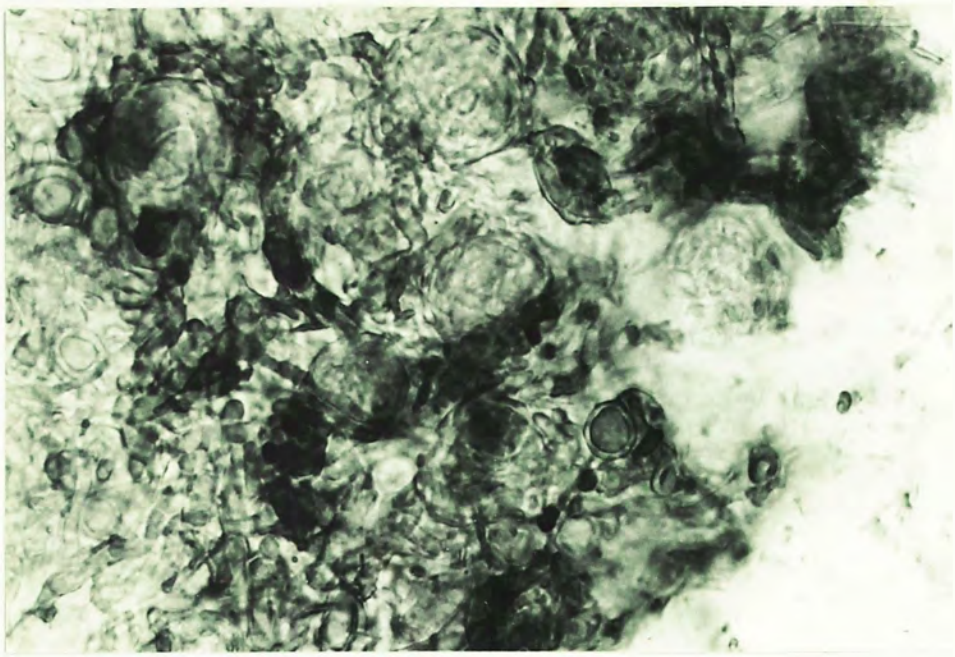
C. Aerial hyphae with chains of conidia. x400.



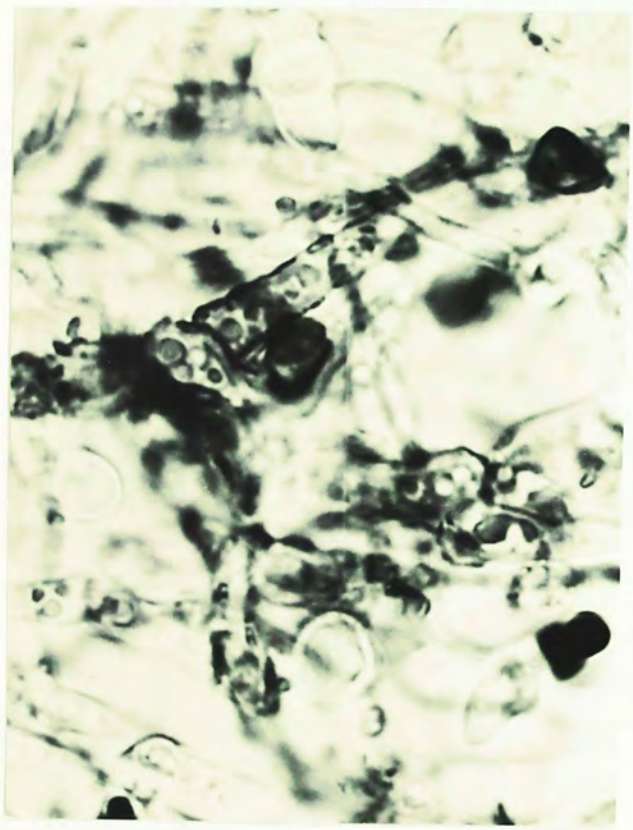
A

Plate 4-3 : Morphology of strain N11S.

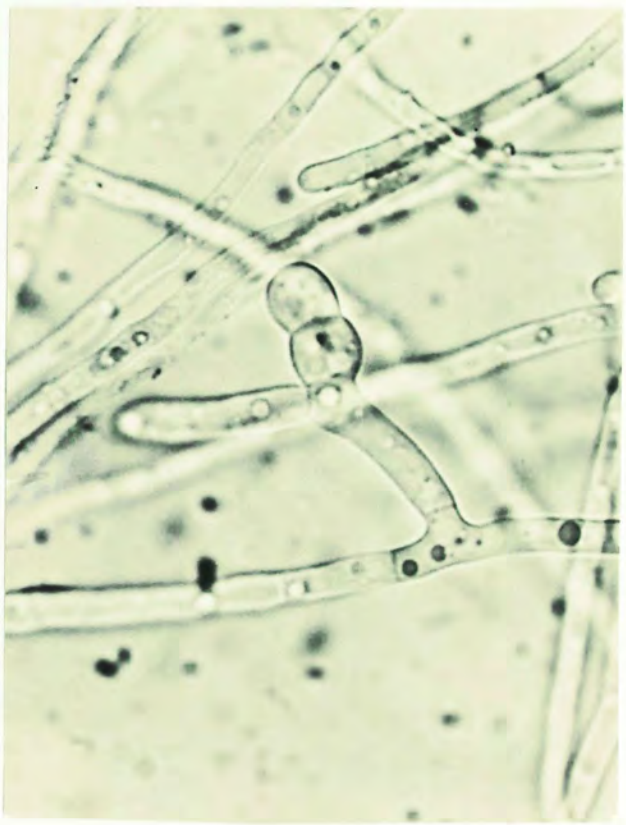
A. 10-day culture. Left, surface view; right, underneath view. x 2/3.



B



C



D

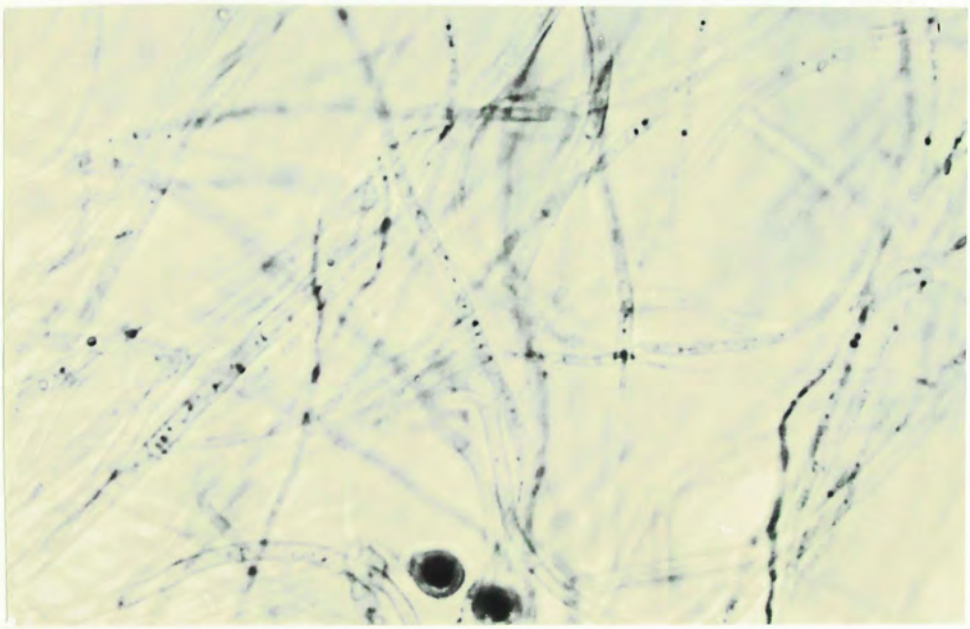
Plate 4-3 (Continued)

B. Mycelium with sterile or protocleistothecia and a few conidia. x 400.

C. Hyphae with pigment surrounding the surface. x 1000.

D. A crozier. x 1000.

A

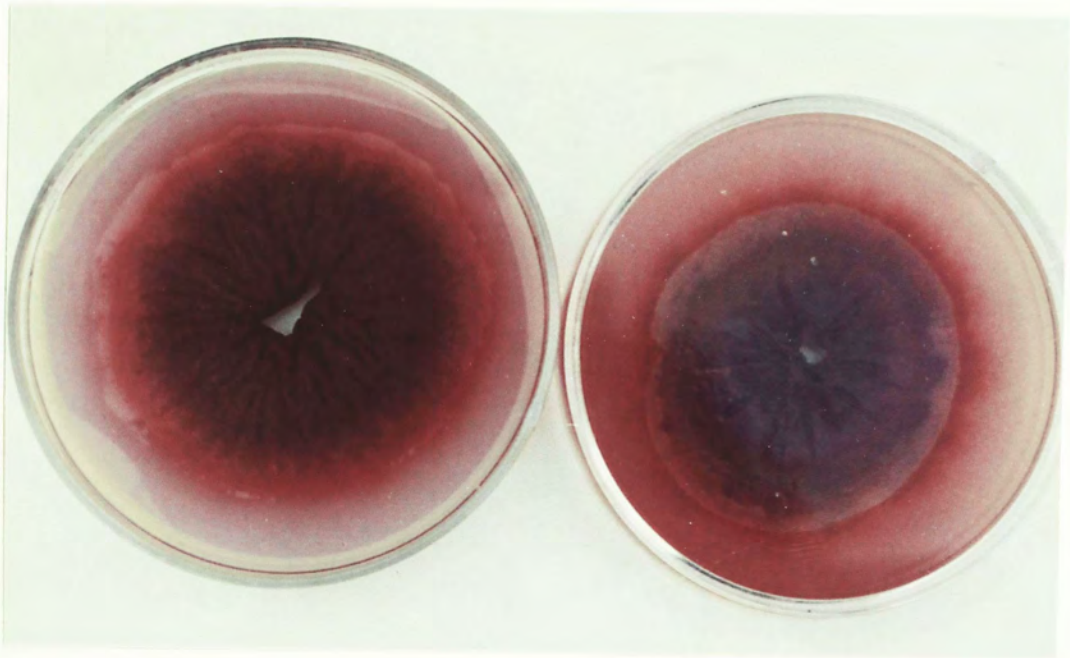


B

Plate 4-4 : Morphology of strain N14S.

A. 10-day culture with furry surface. Left, surface view; right, underneath view. x2/3.

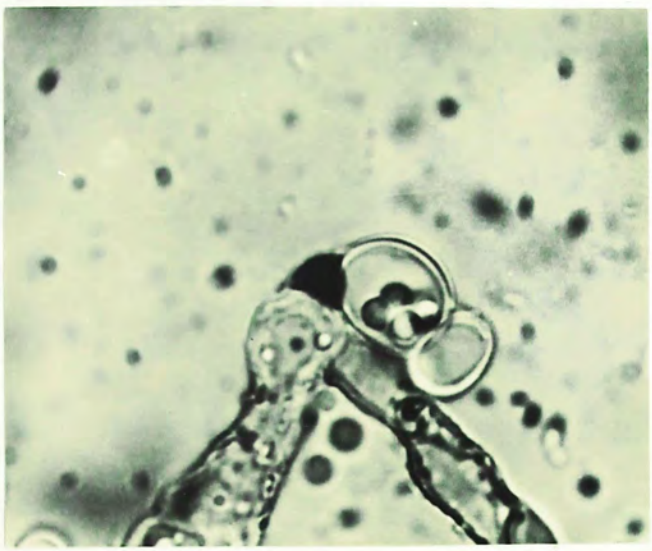
B. Hyphae lacking in pigmentation and with only a few conidia. x 400.



A

Plate 4-5 : Morphology of strain X2P.

A. 10-day culture. Left, surface view; right, underneath view. x 2/3.



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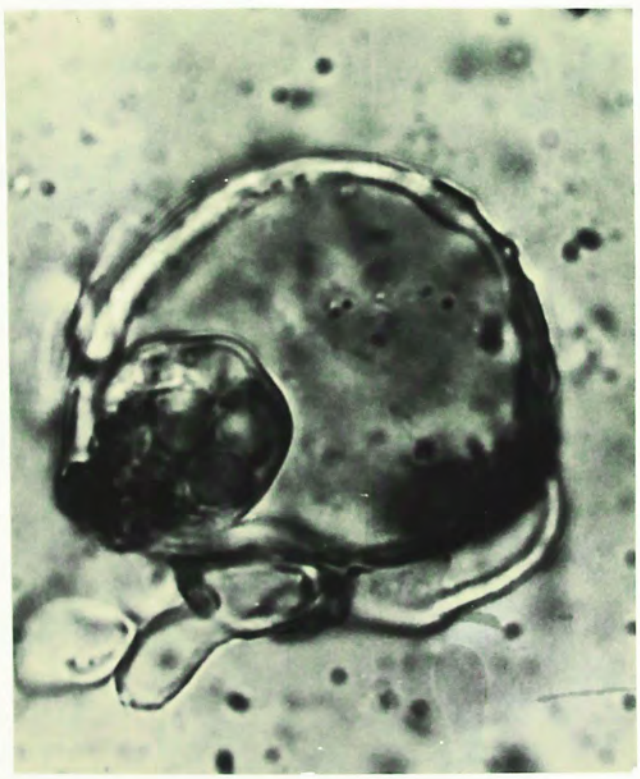
C

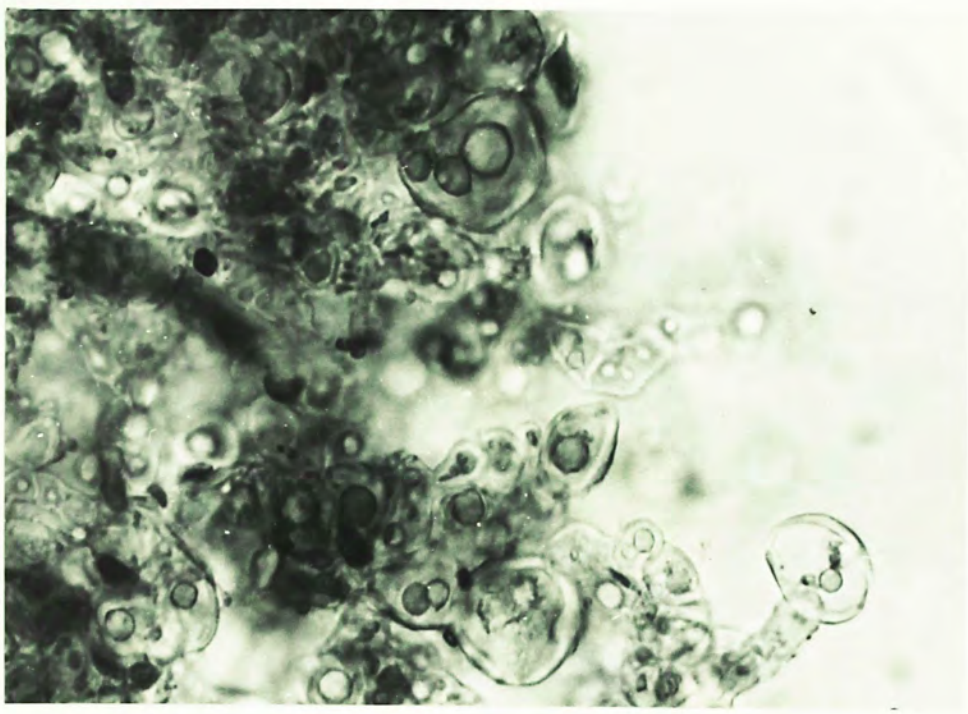


D

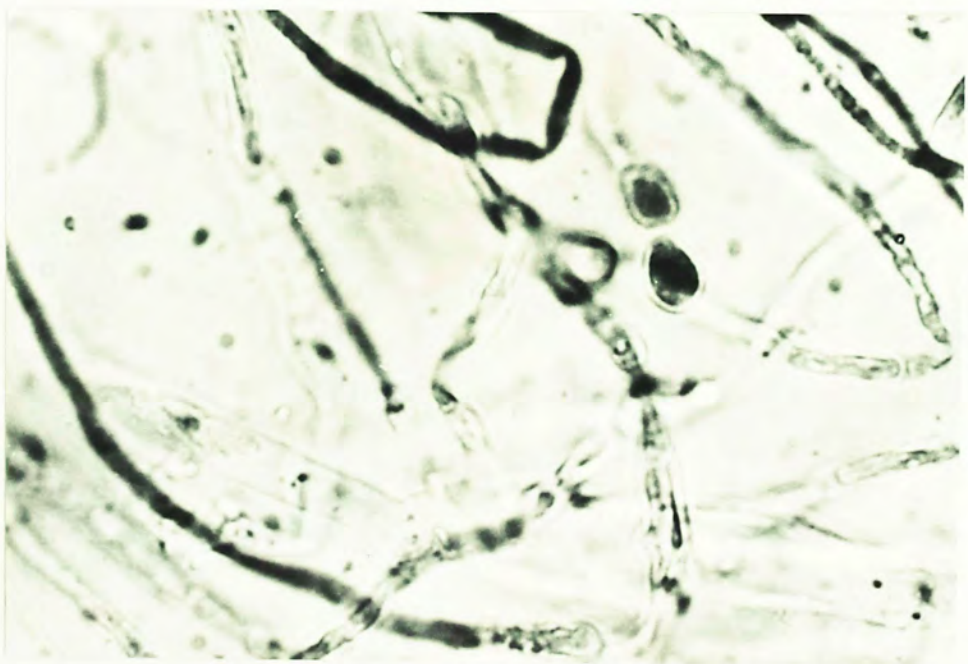
E

Plate 4-5 : (Continued)

B. A crozier. x 1000. C. Young hyphae. x 1000.
D. Cleistothecium with single ascus, large epiplasm and immature ascospores. x 1000.
E. A mature cleistothecium. x 1000.



F



G

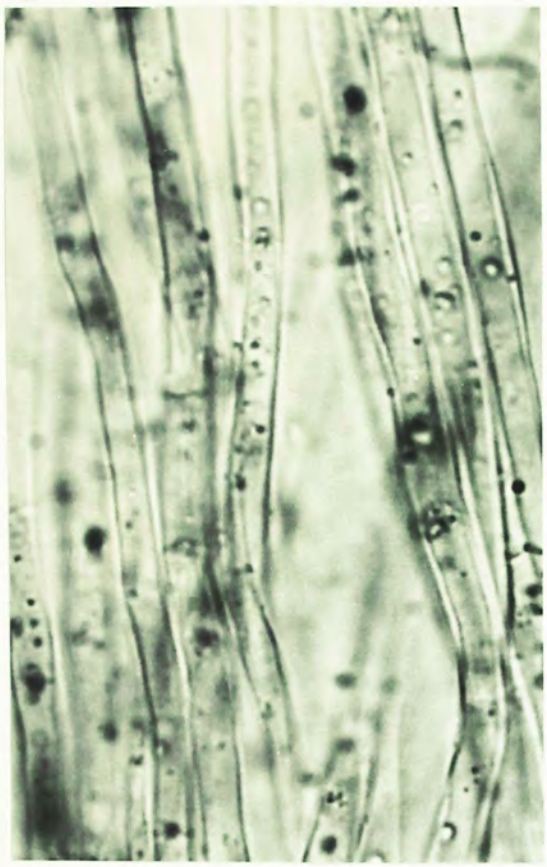
Plate 4-5 (Continued)

F. Abnormal hyphae composed of isodiametric cells with numerous oil droplets. x 1000.

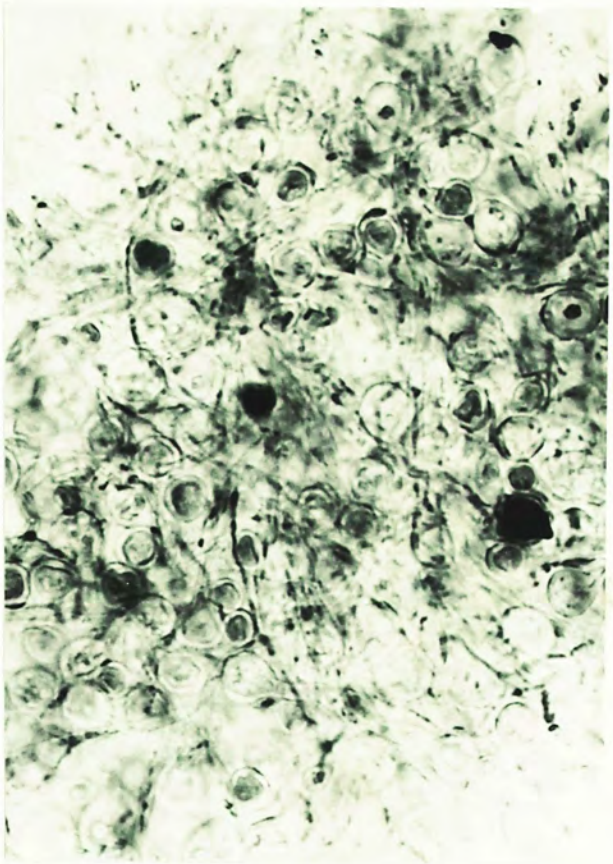
G. Normal filamentous hyphae with elongated cells and few conidia. x 400.

A

Plate 4-6 : Morphology of strain X3S.
A. 10-day culture. Left, surface view; right, underneath view. x 1.



B



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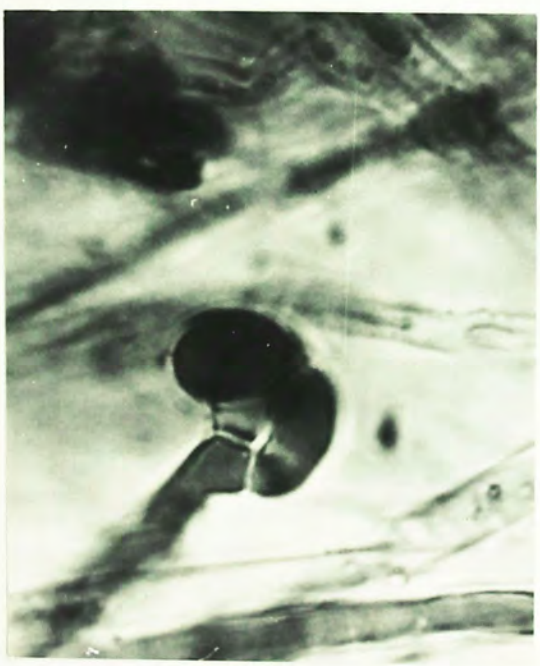

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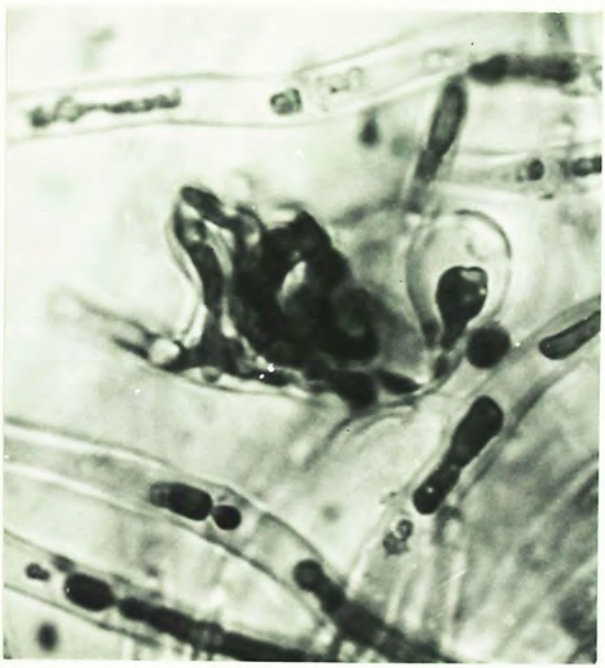
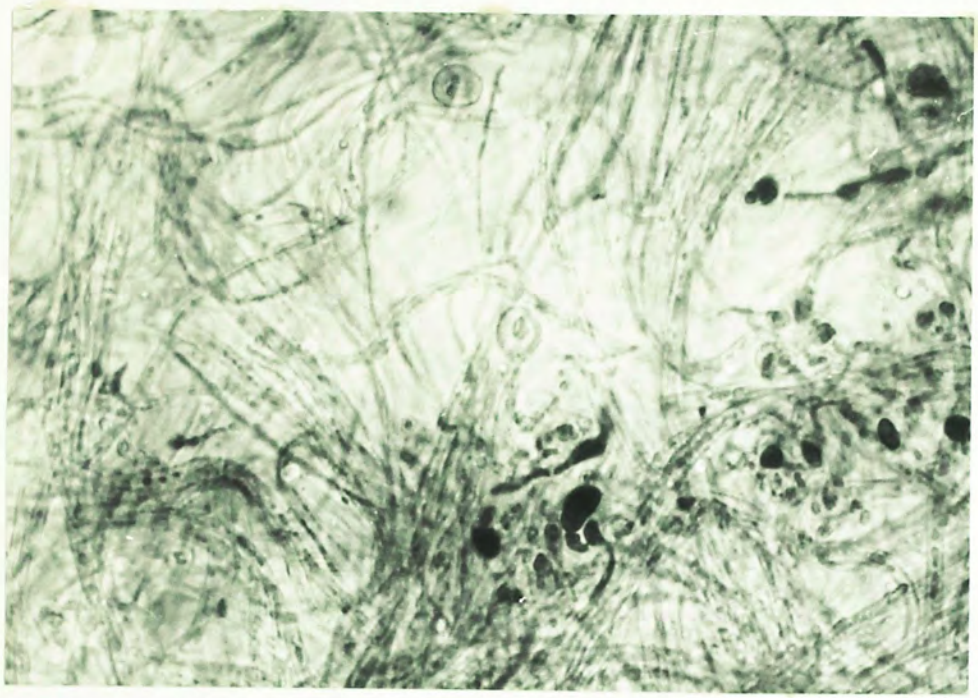


E

Plate 4-6 (Continued)

B. Hyphae with slight pigment. x 1000.
C. Mycelium with numerous conidia. x 400.
D. A crozier. x 1000. E. Cluster of hyphae, no mature cleistothecium. x 1000.

Plate 4-7 : Morphology of strain X4P.
A. 10-day culture. Left, surface view; right, underneath view. x 2/3.



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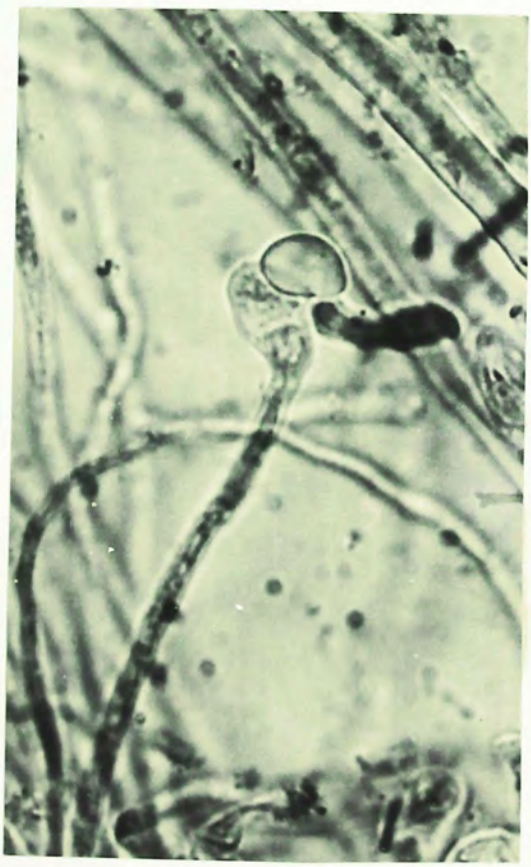

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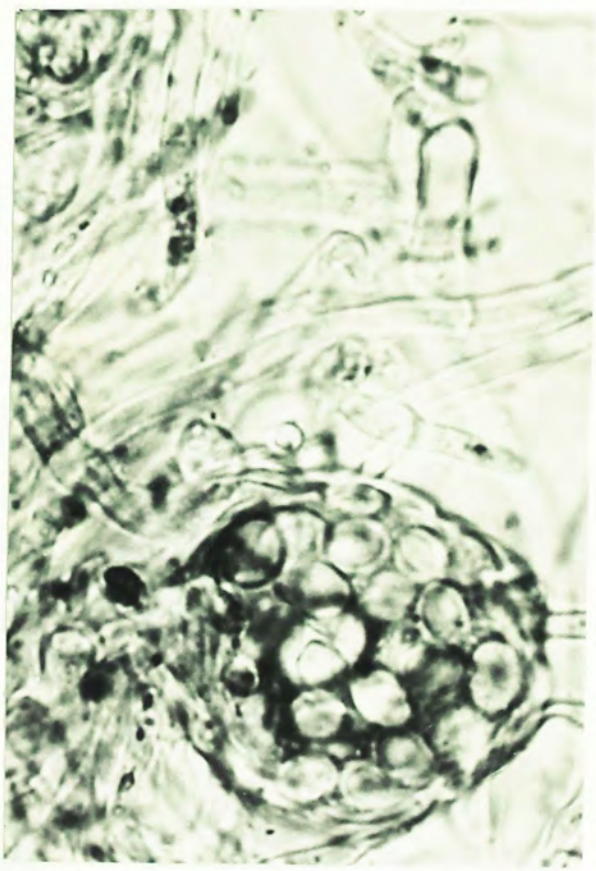


D

Plate 4-7 (Continued)
B. Mycelium with croziers and a few conidia. x 400.
C. A crozier. x 1000. D. A mature cleistothecium with ascospores. x 1000.

Plate 4-8 : Morphology of strain X5S.

A. 10-day culture. Left, surface view; right, underneath view. x 2/3.

B. Mycelium with numerous conidia. x 400.

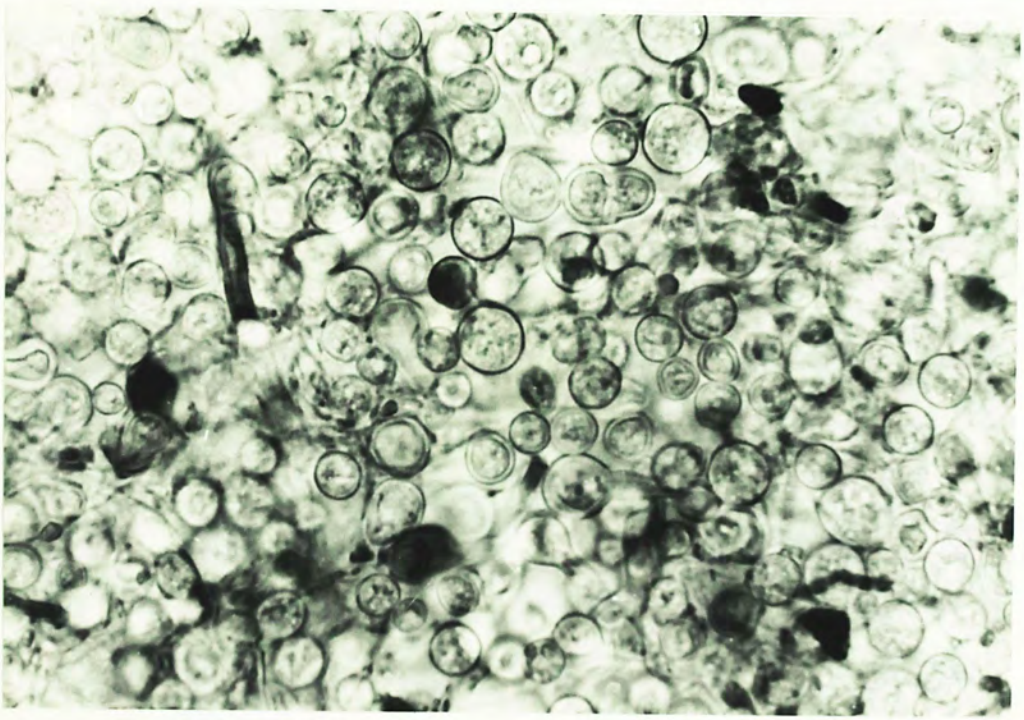
C. An ascogenous hypha, but no crozier has been observed. x 1000.

D. Cluster of hyphae around ascogenous hypha. x 1000.

E. A protocleistothecium.

F. A mature cleistothecium with fewer ascospores. x 1000.

A



B

Plate 4-8 (Continued)

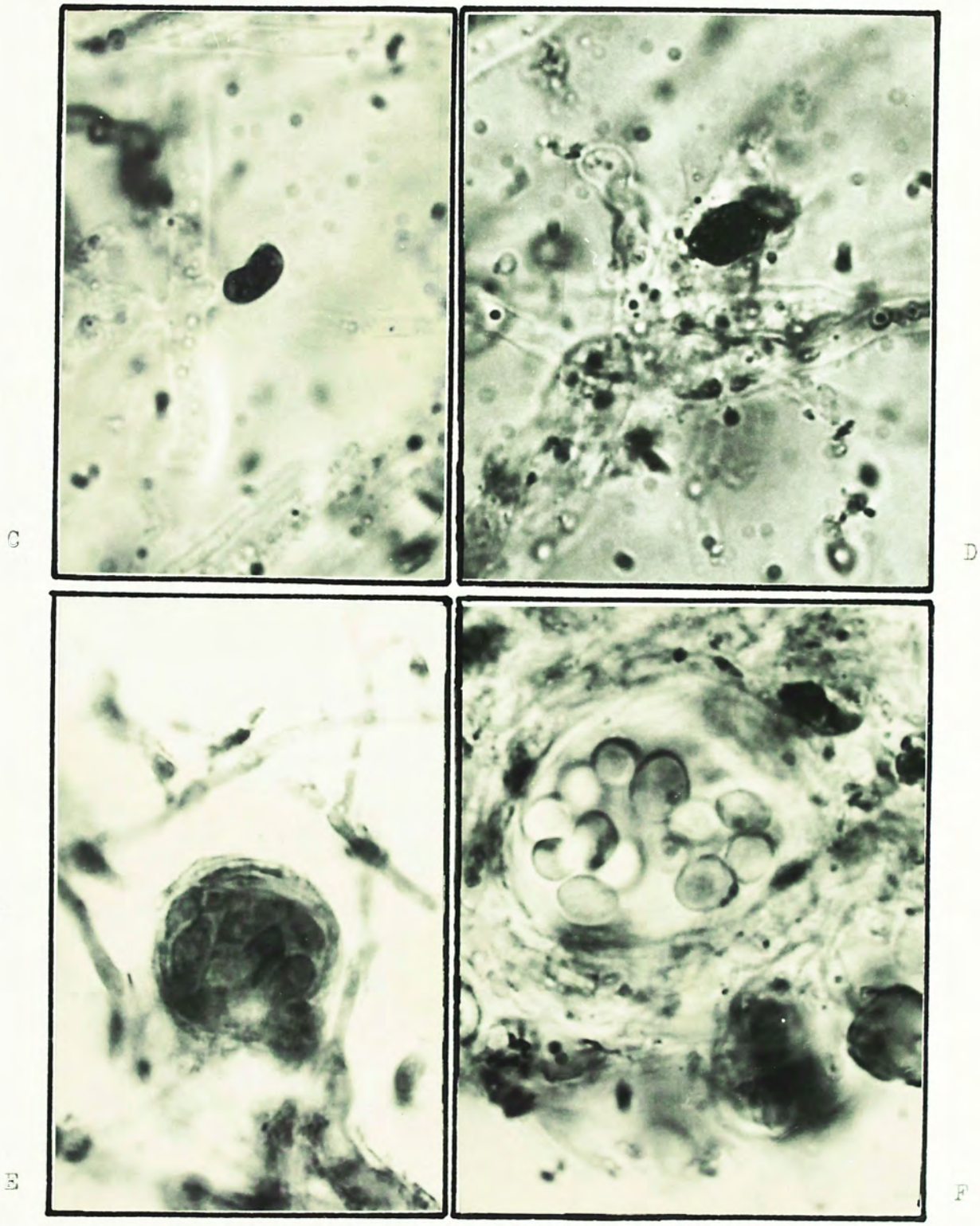
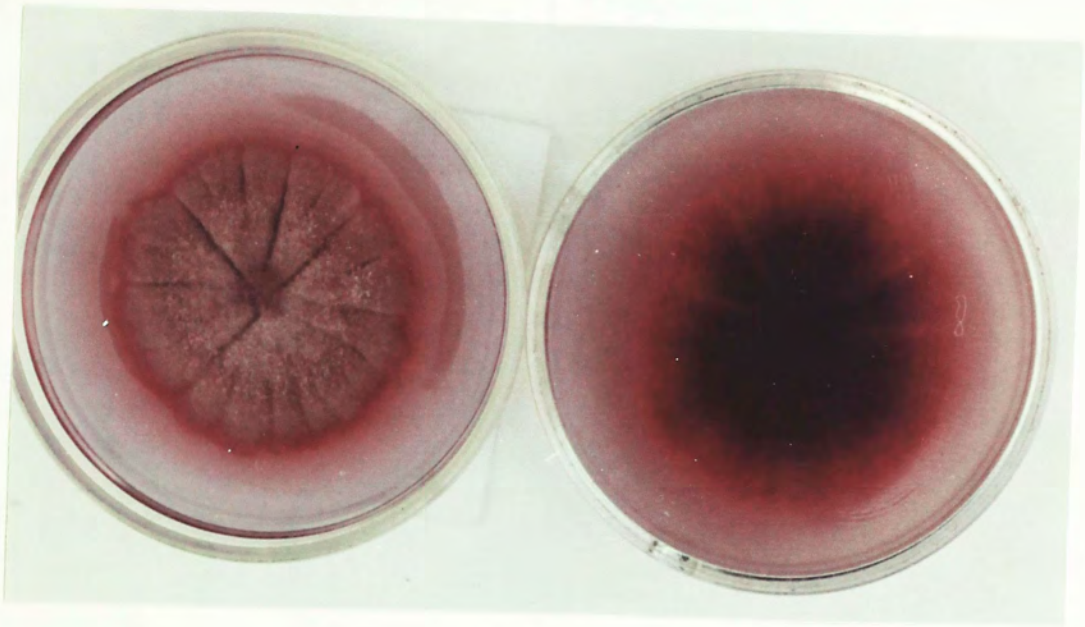


Plate 4-8 (Continued)



A

Plate 4-9 : Morphology of strain L2S.
A. 10-day culture. Left, surface view; right, underneath view. x 2/3.

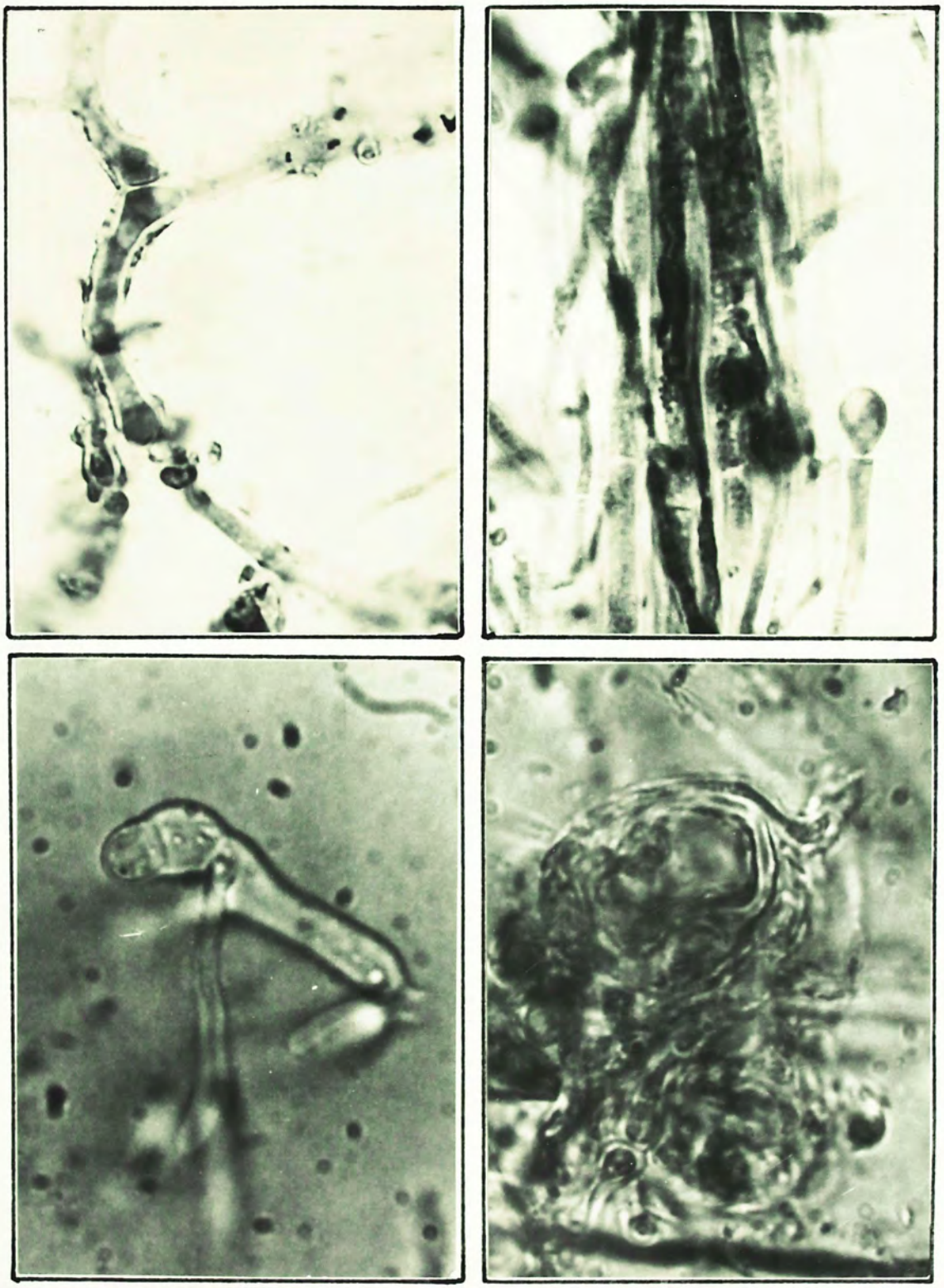


Plate 4-9 (Continued) B. Hyphae with heavy pigment surrounding the surface. x 1000. C. Hyphae with a few conidia. x 1000. D. A crozier. x 1000. E. Sterile or protocleistothecia, without ascospore. x 1000.

CHAPTER FIVE

EFFECTS OF VISIBLE AND ULTRAVIOLET LIGHTS ON FAST-NEUTRON, RUBY LASER AND SOFT X-RAY INDUCED STRAINS OF *MONASCUS PURPUREUS* WENT

Introduction

Photoresponses have been demonstrated in many living organisms, among which photosynthesis may be the most intensively investigated phenomenon. Though the photoresponses of fungi were first discovered at the end of last century, most of the studies were carried out in the last two decades.

The photoresponses of fungi can be grouped into two catagories, the direct responses and the indirect responses (Carlile, 1970). Phototropism (Carlile, 1965) and phototaxis (Bonner and Whitfield, 1965) are direct responses, they occur in minutes or seconds. Other photoresponses which display after hours or even days are indirect responses, such as the pigmentation (Trinci and Banbury, 1969), vegetative growth (Hill, 1976), sporulation (Tan, 1976), spore germination (Leal and Gomez-Miranda, 1965; Lucas et al., 1975) etc.

Effects of different monochromatic radiations to various fungi are not quite similar. However, ultraviolet, blue, red and far-red are the general effective radiations. Sporulation of most light sensitive fungi is stimulated by UV irradiation, for example, conidiation of *Phytophthora* species (Ribeiro et al., 1975) and *Botrytis*

cinerea Pers. ex Fr. (Tan, 1976). Blue light can inhibit conidiation of Botrytis cinerea (Tan, 1975 A and B) and Alternaria tomato (Cooke) Ciferri (Kumagai et al., 1976). On the other hand, blue light stimulates carotenogenesis of Neurospora crassa Shear et Dodge (Fabo et al., 1976), red and far-red are comparatively less active. Far-red stimulates conidiation of Botrytis cinerea, while red and blue reverse the stimulating effects of far-red and near UV (Tan, 1975 A and B). Red light can reactivate and protect Candida guilliermondii (Cast.) Lang et Guerra after UV irradiation, and such effect is abolished to far-red irradiation (Fraikin and Pospelov, 1976). Such photoresponses are somewhat similar to phytochrome regulated phenomenon of green plants.

Photoreceptors responding for various photo-regulated features can be classified into three groups : UV receptors (Leach, 1965; Tan and Epton, 1974), blue light receptors (Munoz and Butler, 1975) and mycochrome (Kumagai, 1973). Mycochrome is a proposed photoreceptor responding for interreversible reactions of different monochromic lights.

Since the knowledge about the biochemical consequences of photoresponses are limited, the mechanism of photoresponses is still far from explicit. The indirect responses may act on the RNA transcription, thus regulating the enzyme pattern, while the direct responses may just act immediately on the enzyme activities (Carlile, 1970).

This chapter reports preliminary study on photoresponses of fast-neutron, ruby laser and soft X-ray induced strains of Monascus purpureus Went.

Materials and Methods

A. Light sources

White light : Periodic illumination of white light was emitted from 500 W mercury light blub (Photolita-NM, Philips). The light passed through a water trough of 7 cm depth. The cultures were placed 33 cm underneath the bottom of water trough. The whole set was installed within a blackened wooden box.

Far-red and blue light : A light filter made up of red and blue cellophane was placed at the bottom of the water trough in the same set described as in white light. The light passed through this filter was far-red.

When a blue plastic filter was placed instead of a far-red device, only blue light passed through.

Red light : Six 40 W fluorescent tubes were wrapped with red cellophane and only red light could pass through. The cultures were placed at 30 cm underneath the light sources.

Ultraviolet light : Ultraviolet light emitted from a UV tube situated inside an inoculation chamber was used. All the cultures were placed at an average distance of 60 cm underneath the UV source.

The intensity of different sources was determined by a photometer, Lightmaster.

Table 5-1 : Intensity of different lights.

Light sources	lux	erg $cm^{-2}sec^{-1}$
White light	11,000	110,000
Blue	2,200	22,000
Red	460	4,600
Far-red	130	1,300
Ultraviolet	54	540

B. Irradiation

All the strains of Monascus purpureus were inoculated to the centers of petri dishes containing 25 ml malt extract agar medium. The cultures were incubated at 35 C in total darkness for 3 days. These young cultures received periodic light irradiations. After the irradiation period, the cultures were kept again in darkness.

C. Estimation of pigmentation

Spectrophotometrical method was used to determine the pigmentation changes regulated by the irradiation period. The 7-day culture, i.e. cultured for 4 more days in darkness after irradiation, were extracted with ethyl acetate, 25 ml per colony, for 24 h. These extracts were filtered through Whatman No. 1 filter paper and air dried. The pigments were diluted to various proportion with 95 % ethanol and the absorbances were determined with a Bausch and Lomb spectronic 70 spectrophotometer at 383 and 510 nm which represented

the absorption maxima of yellow and red pigment respectively.

Results

A. Effect of UV light on various strains

The 3-day cultures of various strains received a 5 min UV irradiation. These cultures were again incubated at 35 C in total darkness. Pigmentations of these UV treated 7-day cultures were determined spectrophotometrically. Spore productions of 10-day cultures were also determined.

The most prominant effect of UV light was the stimulation of pigment synthesis. From Plate 5-1, pigmentations of the slightly colored strains X3S and X4P, and the albino strain N14S were enhanced or restored. The pigmentation was concentrated to the center of the colonies, and only a slight amount diffused to the margin of mycelium pads. Pigments also diffused into the agar medium as shown in the underneath views. When cultures older than 3-day receiving UV irradiation, the pigment usually produced on the young hyphae which received the irradiation. So the photoreceptor might be located in the young hyphae.

Pigmentation of strain X5S was also promoted. In other highly pigmented strains like N11S, X2P and L2S, the pigmentation was slightly inhibited (Table 5-2).

Another morphological change was observed in wild-type, N11S, X2P and L2S, that is the colonies of these strains having a clear bondary separating the older part which had received the UV light and

Table 5-2 : Effect of UV light on the pigmentation of various strains.

Strains	Dilution with 95 % ethanol (ml/colony)	Absorption			
		510 nm		383 nm	
		Control	UV	Control	UV
Wild-type	300	0.36±0.08	0.37±0.04	0.68±0.05	0.65±0.12
N4S	300	0.54±0.06	0.68±0.08 *	1.20±0.10	1.20±0.14
N11S	300	0.65±0.08	0.58±0.08	0.98±0.13	0.74±0.09 *
N14S	5	0.04±0.01	0.34±0.05 **	0.15±0.02	1.20±0.18 **
X2P	100	0.56±0.07	0.41±0.08 *	1.35±0.03	0.75±0.16 **
X3S	15	0.07±0.01	0.47±0.09 **	0.17±0.01	1.24±0.32 **
X4P	5	0.10±0.02	0.17±0.05 *	0.35±0.06	0.74±0.18 **
X5S	100	0.12±0.02	0.29±0.08 **	0.45±0.07	0.97±0.18 **
L2S	300	0.68±0.12	0.51±0.09	1.04±0.12	0.82±0.10 *

*, Significant at P=0.05; **, highly significant at P=0.01.

the newly formed mycelium. Pigmentation within these bondaries were slightly higher (Plate 5-2 A, C, D, and F). These bondaries might be the result of a sudden and short change of growth rates.

In strains N4S and X5S, there was no distinct morphological changes except for a increase in pigmentation.

Spore production of these strains was also affected by UV light. Conidia production of strains N14S and L2S was significantly promoted. Conidiation of N14S was increased from 1.6×10^5 to 4.3×10^5 spores per colony. On the other hand, both sexual and asexual spore production of wild-type and X2P were slightly inhibited (Table 5-3).

Other morphological aspects as described in Chapter Four were also studied, but these differences were not detectable.

Effect of different periods of UV irradiation to pigmentation of strain N14S was investigated. The exposure time ranged from 15 sec to 20 min. The values were means of at least six colonies. Absorbances at 383 nm and 510 nm were determined, since the yellow and the red pigments have absorption peaks at about 383 nm and 510 nm respectively (Su et al., 1973; Chapter Six). Pigmentation increased rapidly and reached maxima at about 5 min. Therefore, 5 min exposure of UV light would saturate the photoresponses of this strain (Fig. 5-1).

B. Effects of different lights on strain N14S

The effects of white, blue, red and far-red lights to N14S were investigated. The exposure time was 1 h for white and 2 h for the others. Pigmentation and conidiation of the irradiated and the control colonies were determined, as shown in Table 5-4.

Plate 5-1 : Morphology of strains N14S (A), X3S (B), and X4P (C), with a 5 min UV irradiation on 3-day cultures. These photographs were taken on day 7. Plates on the left side show surface views, and those on the right show underneath views. x 2/3.

Plate 5-2 : Morphology of wild-type (A), and strains N4S (B), N11S (C), X2P (D), X5S (E), and L2S (F), with a 5 min UV irradiation on 3-day cultures. These photographs were taken on day 7. x 3/2.

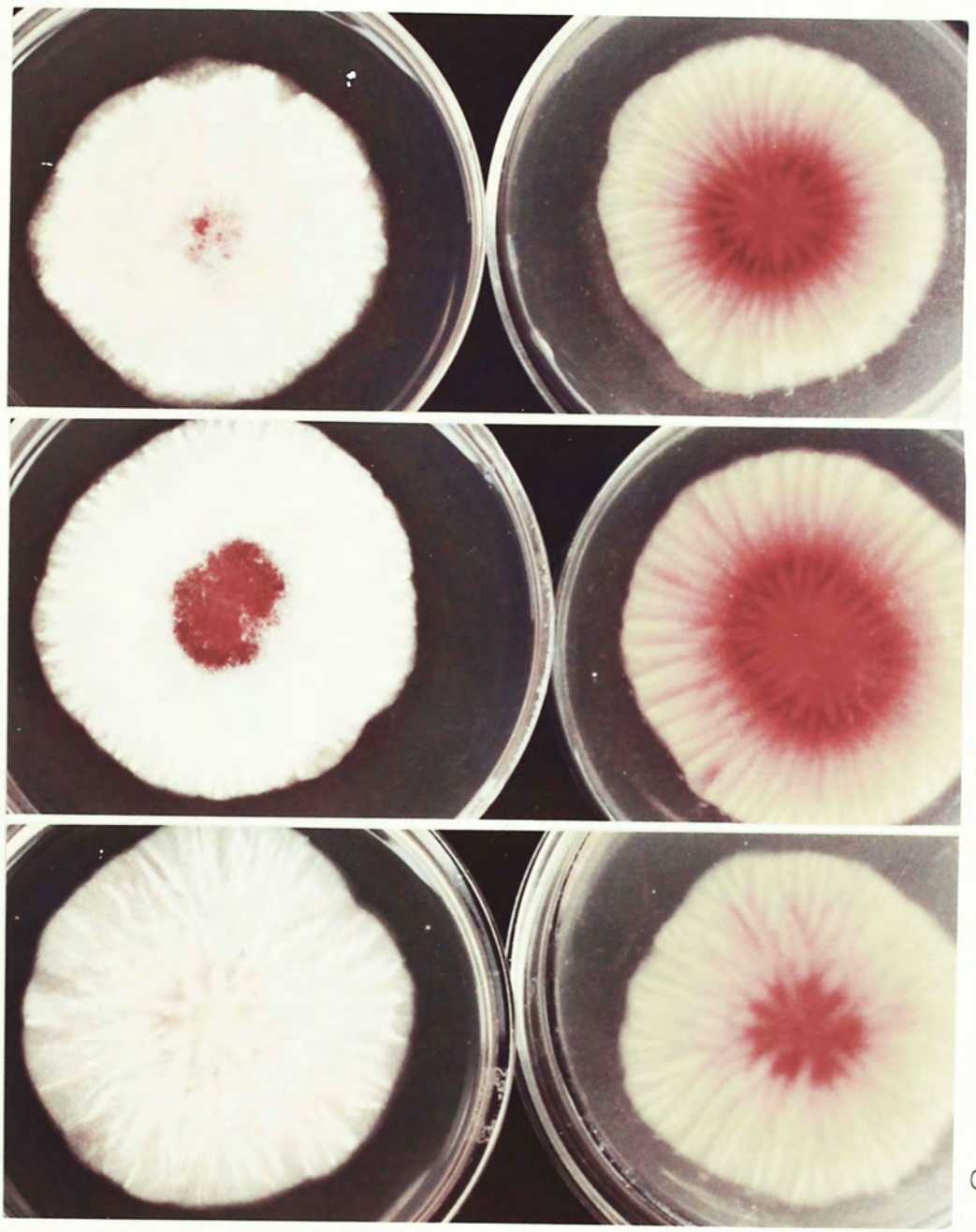


Plate 5-1

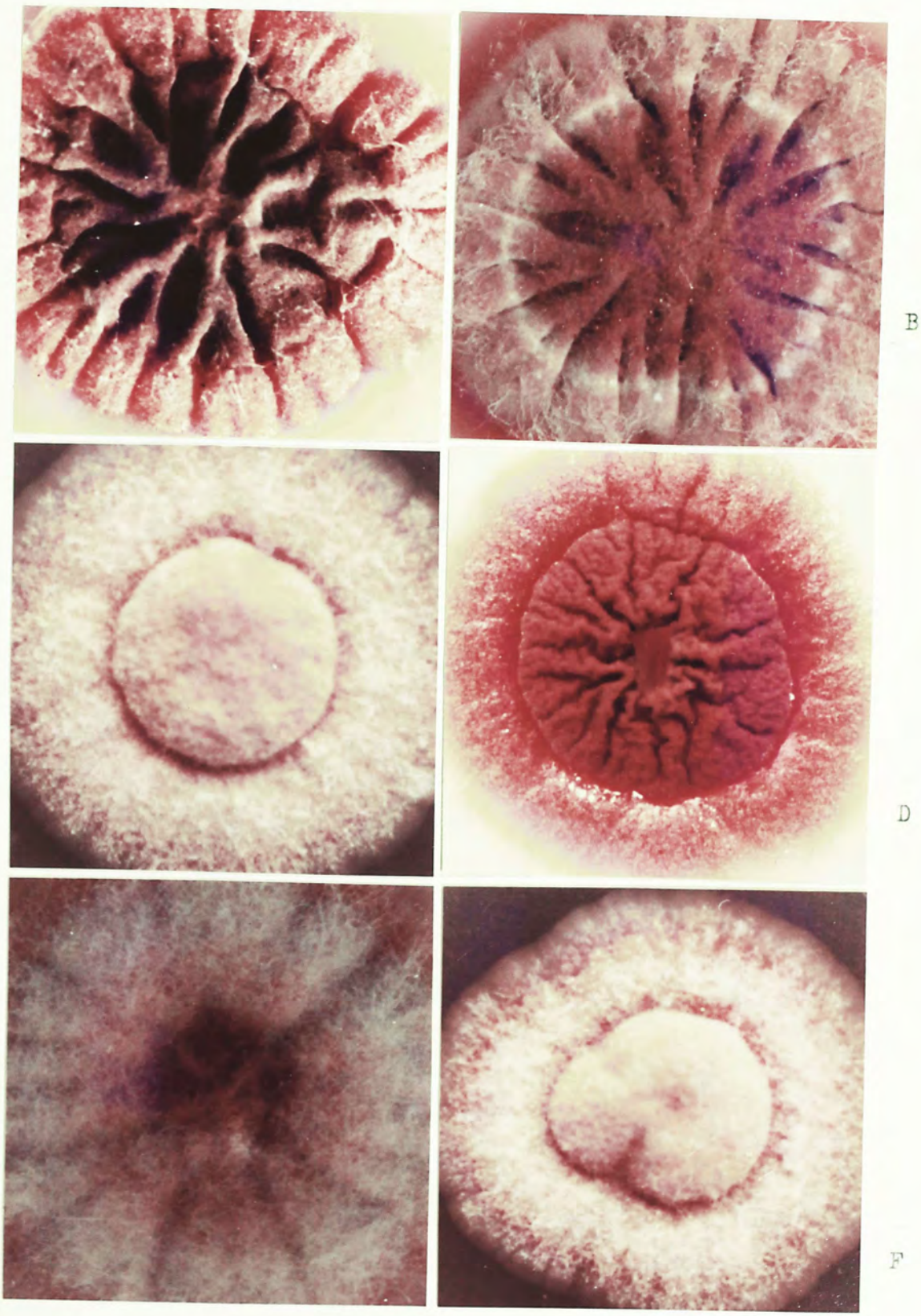


Plate 5-2

Table 5-3 : Effect of UV irradiation on the sporulation of various strains.

Strains	Spores per colony ($x10^5$)			
	Conidium		Ascospore	
	Control	UV	Control	UV
Wild-type	6.4± 3.0	4.9± 1.7	93.0±30.0	64.8±13.0
M4S	98.0±13.0	123.3±32.0	0	0
M11S	0.8± 0.8	1.1± 0.5	0	0
M14S	1.6± 0.7	4.3± 1.7 *	0	0
X2P	2.5± 0.6	0.8± 0.8 **	11.0± 3.0	4.2± 2.7 *
X3S	55.0±14.0	68.1±20.1	0	0
X4P	1.8± 0.4	1.8± 0.2	1.2± 0.2	2.2± 1.2
X5S	150.0±30.0	184.2±30.0	Trace	Trace
L2S	0.5± 0.4	1.2± 0.5 **	0	0

*, Significant at P=0.05; **, highly significant at P=0.01.

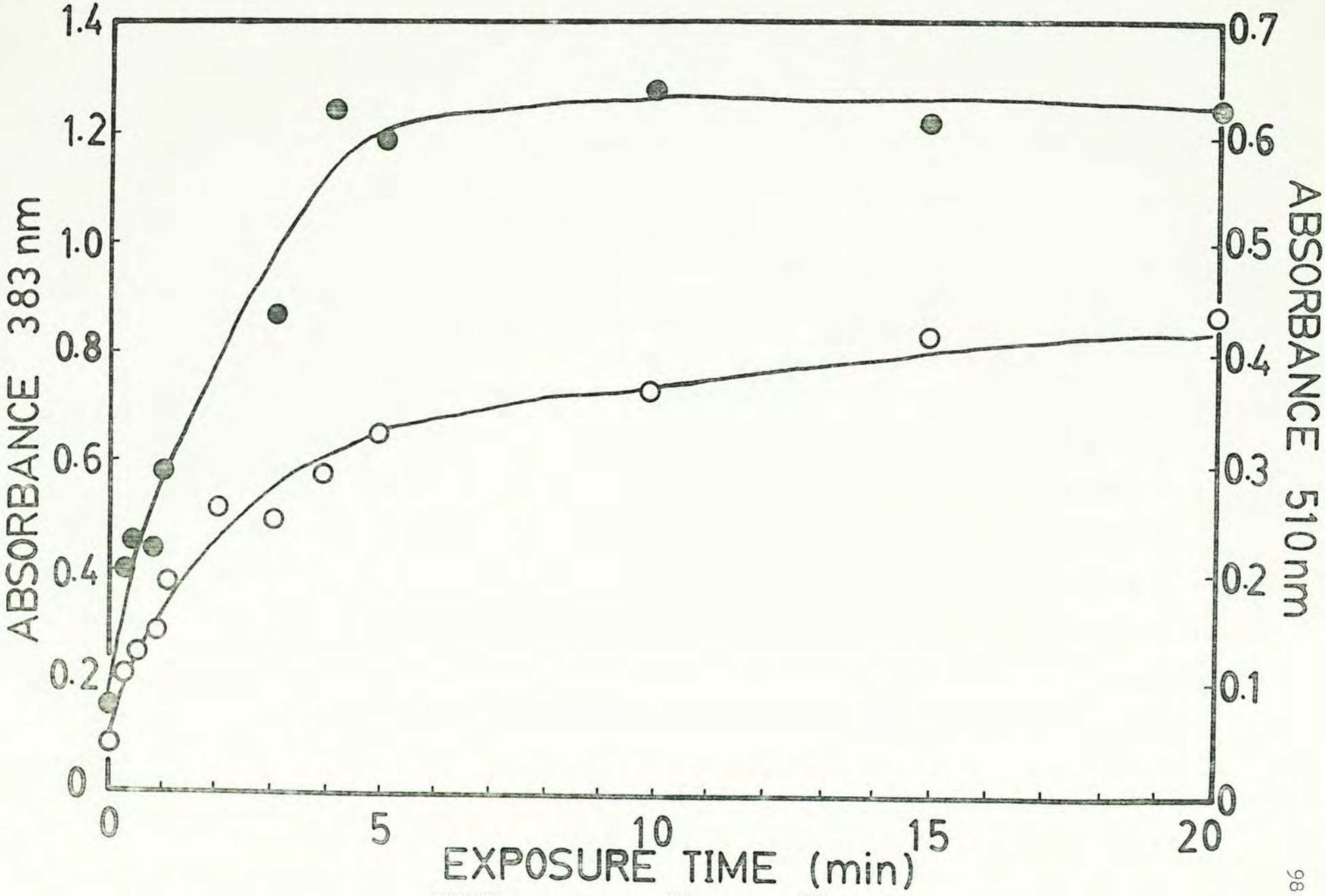


Fig. 5-1 : Effect of different exposure time of UV light to the pigmentation of strain N14S determined spectrophotometrically at 510 nm (○) and 383 nm (●).

Table 5-4 : Effects of white, blue, red and far-red lights on strain N14S.

Lights	10-day cultures		Pigmentation of 7-day cultures	
	Diameter of colony (cm)	Conidiation spores/colony ($\times 10^5$)	510 nm	383 nm
Control	7.0±0.5	1.6±0.7	0.04±0.01	0.15±0.02
White	6.7±0.4	1.1±0.5	0.03±0.01	0.23±0.05 *
Blue	6.7±0.3	1.1±0.4	0.03±0.01	0.22±0.05 *
Red	6.7±0.3	1.5±0.6	0.04±0.01	0.24±0.06 *
Far-red	7.2±0.1	1.4±0.6	0.03±0.01	0.19±0.04

*, Significant at P=0.05.

The different light sources were comparatively inactive to the conidiation and pigmentation of strain N14S. White, blue and red lights slightly promoted the pigmentation as detected at 383 nm but not at 510 nm.

C. Effect of UV light on antibiotic synthesis

Strains N14S, X3S, X4P and X5S did not exhibit any anti-bacterial activity when they were cultured in total darkness. Five min UV irradiation on 3-day cultures of these strains and incubated at 35 C for 4 more days still did not inspire any detectable anti-bacterial activity. In some trials, the 7-day and 10-day cultures of these strains were placed on the window side at room temperature (about 25 C). UV light of intensity about 25 lux was used during night period of about 12 h. Some cultures were wrapped with aluminium foil as control. The anti-bacterial activity was assayed in day 10 after transferring the cultures from incubator to window side (Chapter Six). Growth of all the cultures was limited due to the lowering of temperature. Pigmentation of 7-day cultures was promoted, but not for those of 10-day cultures. It may be the consequence of nutrient exhausted. Anti-bacterial activity was detected from some plates of 7-day culture of strain N14S, X3S and X5S but not of X4P (Table 5-5). All the 10-day cultures did not show any activity of this kind. Further investigation would be required to reveal the interrelationships of pigmentation, antibiotic synthesis and irradiation.

D. Interaction of Bacillus sp. and strain N14S

Bacillus sp. was isolated from the air-borne bacteria in the

Table 5-5 : Antibiotic synthesis of 7-day cultures stimulated by UV and day-light irradiation for 10 days.

	N14S	X3S	X4P	X5S
Control	-	-	-	-
Light	+	+	-	+

+, Present; -, absent.

laboratory (Chapter Six). It was inoculated to the margin of the agar plate on which a N14S colony had already been cultured for 7 days. This plate was again cultured for 3 or more days. When the bacterial colony was near to the fungal colony but not yet met each other, red pigment was produced on the mycelium pad to front facing the bacteria. The color was more deeper as the bacterial colony reached the mycelium, as shown in Plate 5-3.

Discussion

Photoresponses of Monascus purpureus have not yet been demonstrated, so this report might be the first one in this aspect. Wild-type and some of the highly pigmented strains did not respond sharply to UV irradiation, only a small increase or decrease in pigmentatiion were observed. In those slightly pigmented strains such as N14S, X3S, X4P and X5S, the photo-stimulated pigmentation was extremely effective.

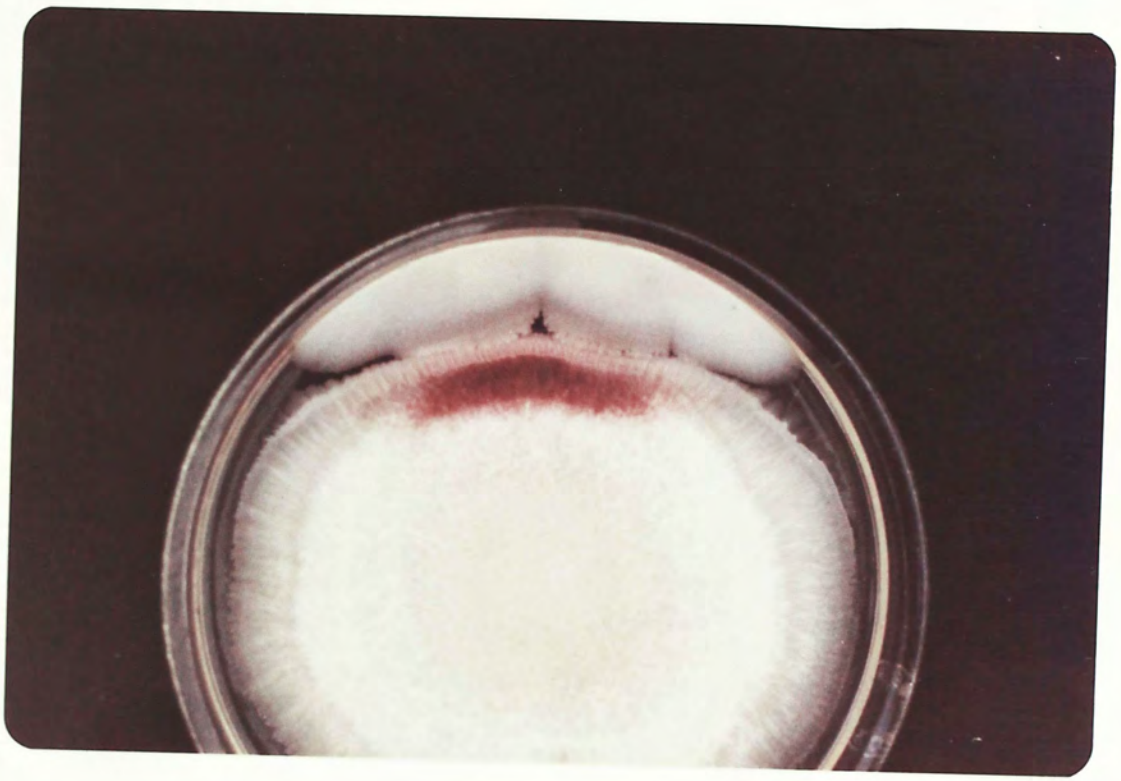


Plate 5-3 : Pigmentation of strain N14S stimulated by *Bacillus* sp. *Bacillus* was inoculated to the margin of plate with 7-day N14S colony and cultured for 4 more days.

Among these strains, N14S and X3S were the most sensitive ones. As determined at 510 and 383 nm, an increase of 7 times was produced even by a short period of 5 min exposure to UV light. Nearly double pigmentation was observed in strain X4P and X5S.

Photo-regulated pigmentation of fungi is not an extraordinary phenomenon, but the study of photoresponses of fungi generally complies with sporulation. Carotenoid, which is believed to be one of the photoreceptors and protectant against photo-destruction, is the main pigment studied (Fabo et al., 1976). Pigments other than carotenoids studied are naphothoquinone, melanin, etc. (Carlile, 1970). The photo-depression or stimulation of these pigments in different species may not be identical. Action spectrum for the photoinduction of carotenoid synthesis in Neurospora crassa showed peaks at 450 and 481 nm which are blue lights. Though the UV portion is also effective, the response is fairly low. But in the photoinduced pigmentation of Monascus purpureus, UV light was the most effective one, where white, blue, red and far-red were ineffective even though much higher intensities and exposure time were used. This result is quite different from other studies. In some cases, UV light stimulates sporulation (Carlile, 1965 and 1970), but not pigmentation. The effect demonstrated in this study was likely to be a "low energy" effect, since a exposure of 5 min UV light would obtain a saturated response.

Conidiation was also stimulated by UV light, especially in strain N14S. Ascospore production of the strains was not stimulated. The response of spore production of various strains was quite different.

The metabolism affected by light has been investigated. Light is known to inhibit growth, glucose uptake and phosphorylation of Aspergillus ornatus Raper et al. leading to conidiation (Hill, 1976). But most of these studies do not reveal the very initial biochemical reactions. So the study of photoreactions to fungi usually obtain indirect evidence, such as the action spectra (Fabo et al., 1976; Sproston, 1971), the isolation of photoreceptors (Munoz and Butler, 1975; Poff and Butler, 1974; Poff et al., 1974), and the postulation of action models (Fabo et al., 1976; Kumagai, 1973; Tan, 1975 A).

P310 was proposed to be a UV photoreceptor (Leach, 1965), but there were some contradictory results about it's nature and function (Trione et al., 1966). In the present study, a photoreceptor similar to some redox dyes which had been demonstrated to be exogenous photoreceptors (Lang-Feulner and Rau, 1975) is proposed.

One or more of the pigments in M. purpureus might act as photoreceptors. The yellow pigment is supposed to be one of them, because : (1) This pigment has absorption maxima at 383 nm and some shorter wavelengths, and no absorption peak across the blue and longer wavelength. Blue light, red and far-red lights were shown to be almost ineffective to strain N14S. (2) It has been shown that this pigment can be oxidized to rubropunctatin (Chen et al., 1971). This reaction may be reversible in vivo as stimulated by lights. Redox dyes such as methylene blue, toluidine blue, and neutral red can act as photorecptors in inducing carotenoid synthesis in Fusarium aquaeductuum Lagerh. (Lang-Feulner and Rau, 1975). (3) In strain N14S, only slightly yellow color occurred in the ethyl acetate extracts of dark grown cultures.

Since the photo-stimulated pigmentation of Monascus purpureus took place in the young mycelium, two models can be proposed to explain the photorecations. The first one, Model 1, consists one compound (R) which located in the young hyphae only and would react with the activated photoreceptor (P_A) to form an intermediate compound (P-R) which was involved in the pigment synthesis. Model 2 consists no such compound, therefore the photoactivated photorecptor (P_A) directly involved in the pigment synthesis (Fig. 5-2).

The compound(s) produced by Bacillus sp. might be the activated photoreceptor (P_A) in both models, or the intermediate compound (P-R) in model 1. Since the photoresponses of strain N14S could be easily saturated, the compound R in model 1 or the photoreceptor in both models would be limiting factors.

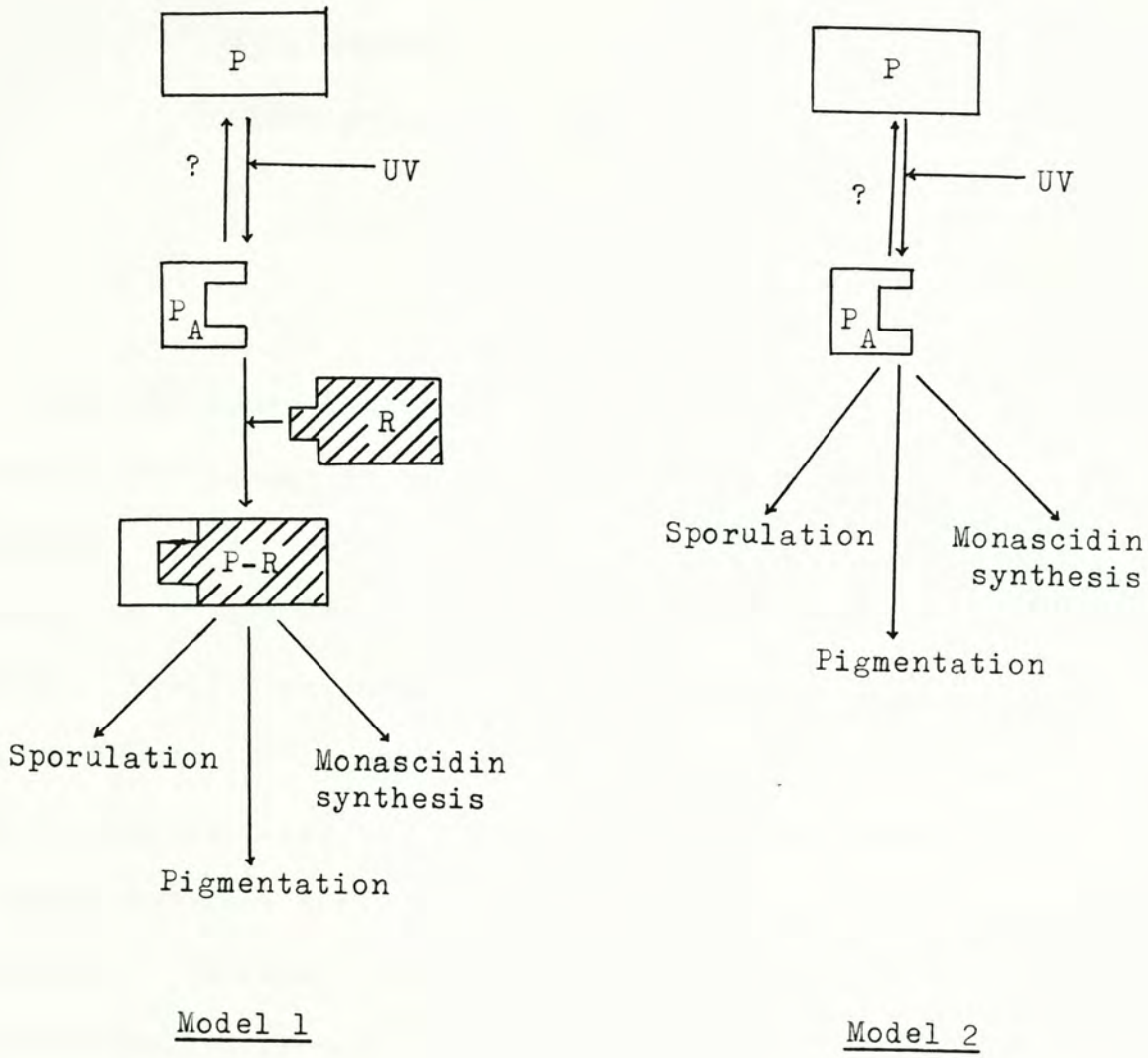


Fig. 5-2 : Models to explain photoresponses of Monascus purpureus N14S. P, photoreceptor; P_A, photoactivated photoreceptor; P-R, intermediate compound; R, compound existed in young hyphae only; UV, ultraviolet irradiation.

CHAPTER SIX

PIGMENTATION AND ANTI-BACTERIAL ACTIVITY OF FAST-NEUTRON, RUBY LASER AND SOFT X-RAY INDUCED STRAINS OF *MONASCUS PURPUREUS* WENT

Introduction

The antibiotic activities of various organisms have been screened. The common antibiotic producers are : actinomycetes (Broadbent, 1966), bacteria (Gauthier and Flatau, 1976; Hsu and Wiseman, 1967; Vaks et al., 1974), and fungi (Allbutt et al., 1971; Stillwell, 1966). The antibiotic activities of fungi have been reviewed (Brian, 1951; Broadbent, 1966). A number of fungi were shown to produce certain activities while others were not. Still, one cannot conclude that certain group of related fungi produces antibiotics. There are two difficulties; firstly, fungi have not been thoroughly screened for such activities, and secondly, specific indicator organism may be required.

Antibiotic activities of fungi have been demonstrated in phycomycetes, ascomycetes, basidiomycetes, and deuteromycetes. The distribution of active species are not even, this may be a result of unbalanced screening of species from each group of fungi. Moniliales and Agaricales were reported to produce more antibiotics (Broadbent, 1966). *Monascus* sp. was shown to be inactive (Brian, 1951). The activities of some antibiotics are affected by pH.

Although a lot of fungi have been shown to be active, the chemistry of active compounds has not been clearly revealed. Some of them may be identical compounds, with different postulated structures. The molecular structures may be simple, such as hydrogen peroxide (LeBien, 1975) or very complex like polypeptides, but most of them are aromatic compounds (Brian, 1951; Strunz et al., 1969).

Not all the antibiotics have practical values, only a few are used chemotherapeutically. Recently the seeking of anti-cancer antibiotics gains unusually popular attentions.

The pigmentation of Monascus purpureus has been investigated, as mentioned in Chapter One. This chapter provides a comparison of the pigmentation of different strains, quantitatively and qualitatively with special reference to the anti-bacterial activities. It is also the first report of this kind in this fungus.

Materials and Methods

A. Extraction and isolation of pigments

All the strains were inoculated in the center of petri dishes containing 25 ml malt extract agar and then incubated at 35 C for 10 days. Some colonies were extracted with 25 ml ethyl acetate for 24 h at room temperature and the filtrate air dried. Other colonies were extracted with 25 ml petroleum ether (b.p. 60-80 C) and also air dried. The dry weight of the crude extracts were determined. Thin layer chromatography (TLC) was used to examine

the quality of pigments from various strains. The concentrated crude extracts were applied to 0.25 mm thick silica gel thin layer chromatograms (Merck) and developed with benzene:chloroform:methanol =85:12:3 v/v/v (Chen et al., 1971). The visible and UV absorption spectra of different pigments were determined with a Beckman 25 spectrophotometer.

B. Bioassay of anti-bacterial activity

About 20 ml of nutrient agar medium containing 1.5 % agar was dispensed into sterile 9-cm petri dishes. Later, three ml of 45 C medium containing 0.75 % agar and an indicator bacterium was poured onto this hard agar surface (Slater et al., 1971). Unknown substances were tested from blocks removed from the vicinity of the fungal colonies (0.6 cm in diameter and 0.3 cm thick), from paper chromatogram strips, and from impregnated filter paper discs (0.6 cm in diameter) by placing them on the surface of indicator plates and incubating at 35 C for 12 h (Hash, 1975). One Bacillus species collected from air-borne bacteria in the laboratory was used as an indicator as well as 15 additional species of bacteria.

C. Isolation of anti-bacterial compound(s)

The crude extracts obtained from Section A were spotted on TLC and developed with solvent composed of benzene, chloroform, and methanol in various proportions. The chromatograms were observed under UV light and the fluorescence and other colored bands dissolved in ethanol. Filter paper disc impregnated with these compounds were placed on indicator plates to test for anti-bacterial activity. Paper chromatography was also used. Whatman No. 1 chromatography paper was cut into 1.2 x 20 cm strips. After developing with various solvents, the chromatograms were dried, cut in

half, and placed on indicator plates to detect anti-bacterial activity.

The visible and the UV absorption spectra of an active compound were also determined.

Results

A. Quantitative and qualitative analysis of pigmentation

Since strain N14S was practically albino, it was excluded in the study of pigmentation. The fraction extracted from the remaining strains by petroleum ether (b.p. 60-80 C) was yellow-colored while the ethyl acetate fractions contained all the red pigments. From Table 6-1, strains N4S, N11S, X2P and L2S were rich in yellow pigments, having about twice as much as the wild-type. The total amounts of yellow and red pigments extracted by ethyl acetate from N4S, N11S and L2S were about twice as much as from the wild-type.

The TLC study revealed that the quality of pigments extracted from these strains was the same as for the wild-type except for N11S and L2S. N11S and L2S pigments extracted by petroleum ether were golden yellow in color and were separated into two major bands on TLC, one yellow and the other orange (Y2). The orange pigment ran close to the yellow band (Fig. 6-1 and Plate 6-1). Thus the purification of this pigment from the TLC conditions used in this study may not be satisfactory. This pigment was apparently unstable because it was bleached by exposure to air overnight. This orange pigment was unstable in alcohols like methanol, ethanol, butanol,

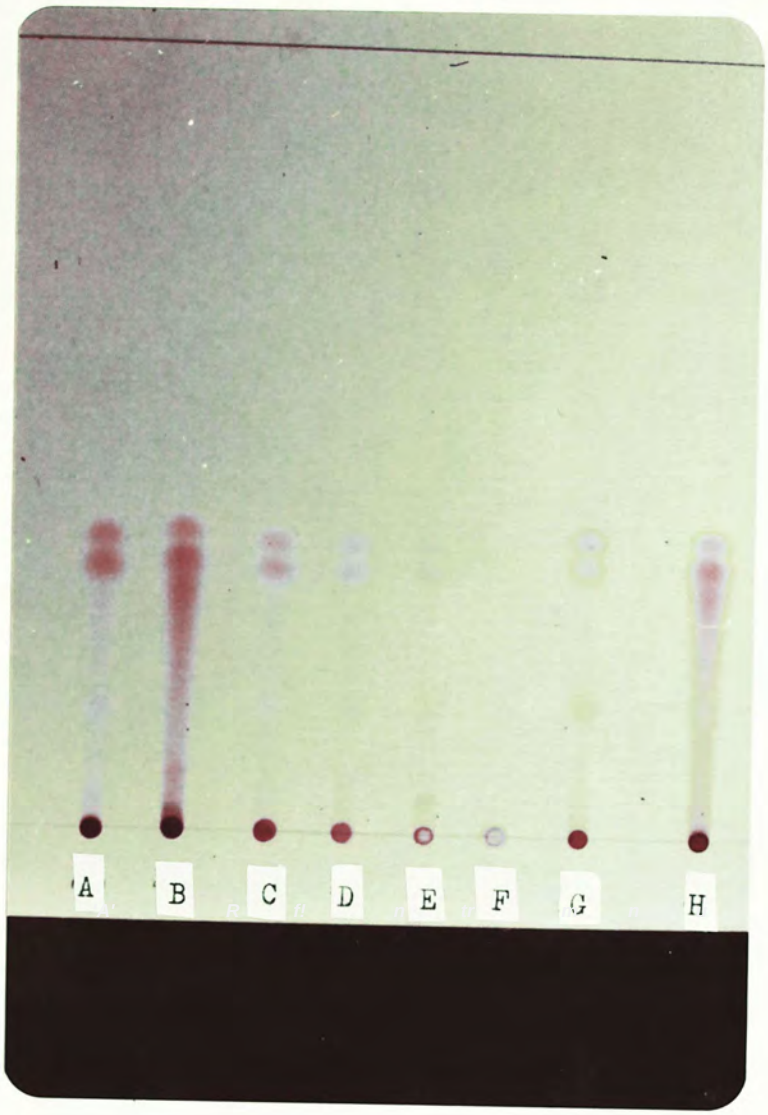


Plate 6-1 : TLC of pigments from various strains.
The ethyl acetate fractions were spotted and developed with benzene:chloroform:methanol= 85:12:3. Yellow pigment ran ahead of others and separated into two bands but of identical absorption spectra. Orange pigment found in strains N11S and L2S just followed the yellow. A, N4S; B, N11S; C, wild-type; D, X2P; E, X3S; F, X4P; G, X5S; H, L2S. The line on the top shows the solvent front.

and even with malt extract agar to form a red pigment which appeared to be identical to the red pigment found in all strains including wild-type (Plate 6-2).

Table 6-1 : Quantity of pigments from various strains of Monascus purpureus extracted by petroleum ether and ethyl acetate.

Strains	Extracted by petroleum ether (mg/colony)+	Extracted by ethyl acetate (mg/colony)+
Wild-type	1.4±0.5	11.1±1.5
N4S	3.7±0.5 **	20.7±2.0 **
N11S	3.0±0.3 **	21.3±1.8 **
X2P	2.4±0.3 *	9.6±1.1
X3S	1.1±0.5	3.2±1.3 **
X4P	0.9±0.1	3.0±0.7 **
X5S	1.0±0.4	6.1±0.7 **
L2S	3.2±0.4 **	18.9±4.0 **

+, The values are the means of 6 determinations.

*, significant at P=0.05; **, highly significant at P=0.01.

The red pigment found in these strains could be resolved into several clearly independent bands with a more polar solvent (benzene:methanol:chloroform=30:10:9) and silica gel (Fig. 6-1). These bands were numbered as R1, R2, R3 and R4 according to their

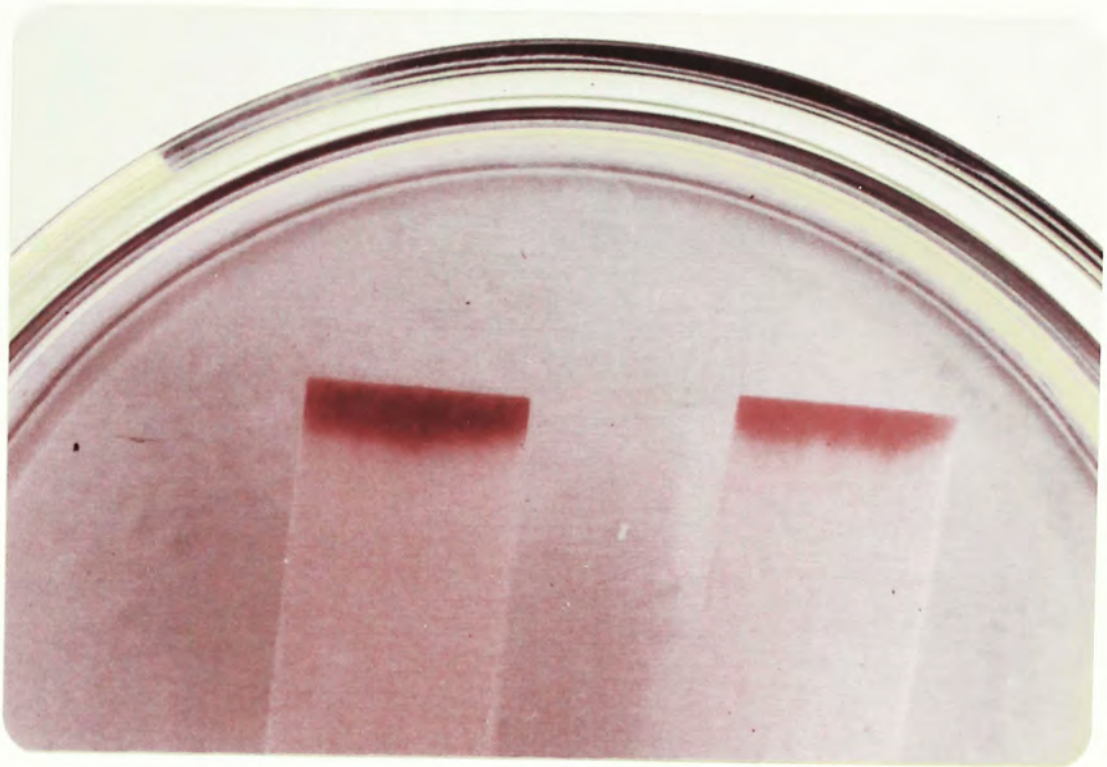


Plate 6-2 : Change of orange pigment (Y2) to red pigment on malt extract agar overnight at 35 C. The petroleum ether fraction was developed on paper chromatography with solvent, benzene:chloroform:methanol=85:12:3. The yellow and the orange pigments ran to the solvent front. Strip on the right was just dried after development, and the left had been incubated on the agar medium for 12 h. On TLC, only the orange pigment changed to red.

Rf values (Table 6-2). There were also some minor bands just above R4, but they were faint and not clearly separated. The UV and visible absorption spectra of R1 and R2 were identical as were the spectra from R3 and R4. The R1 and the R2 spectra had maxima at 500, 362, 288, 263, 257, and 251 nm while the maxima from R3 and R4 were at 515, 263, 257, and 251 nm (Fig. 6-2). The absorption spectra of the yellow pigment, orange pigment (Y2) and the unknown pigments (Y1 and Y3) visible under UV light were also determined. Since Y3 ran quite close to Y2, its absorption spectrum might come from an impure mixtures. Y1 had UV absorption spectrum quite similar to other red pigments. The absorption spectrum of the orange pigment (Y2) appeared to have no maxima, but with two shoulders at about 270 and 480 nm (Fig. 6-3). The Y3 existed in both the petroleum ether and the ethyl acetate fractions while Y1 was only found in the petroleum ether fraction (Fig. 6-1). Y1 was clearly separated on TLC and was apparently pure. Its UV absorption spectrum was similar to that of the red pigments (Fig. 6-3).

B. Anti-bacterial activity

The anti-bacterial activity of the strains was evaluated by measuring the diameter of inhibition zones (Table 6-3). The anti-bacterial activities of various strains were quite variable. Strain N11S had a higher anti-bacterial activity and N4S was slightly lower as compared to the wild-type. Strains with low pigmentation like N14S, X3S, and X4P and the moderately pigmented strain X5S were inactive against the indicator bacterium.

The accumulation of antibiotics in the agar medium also in-

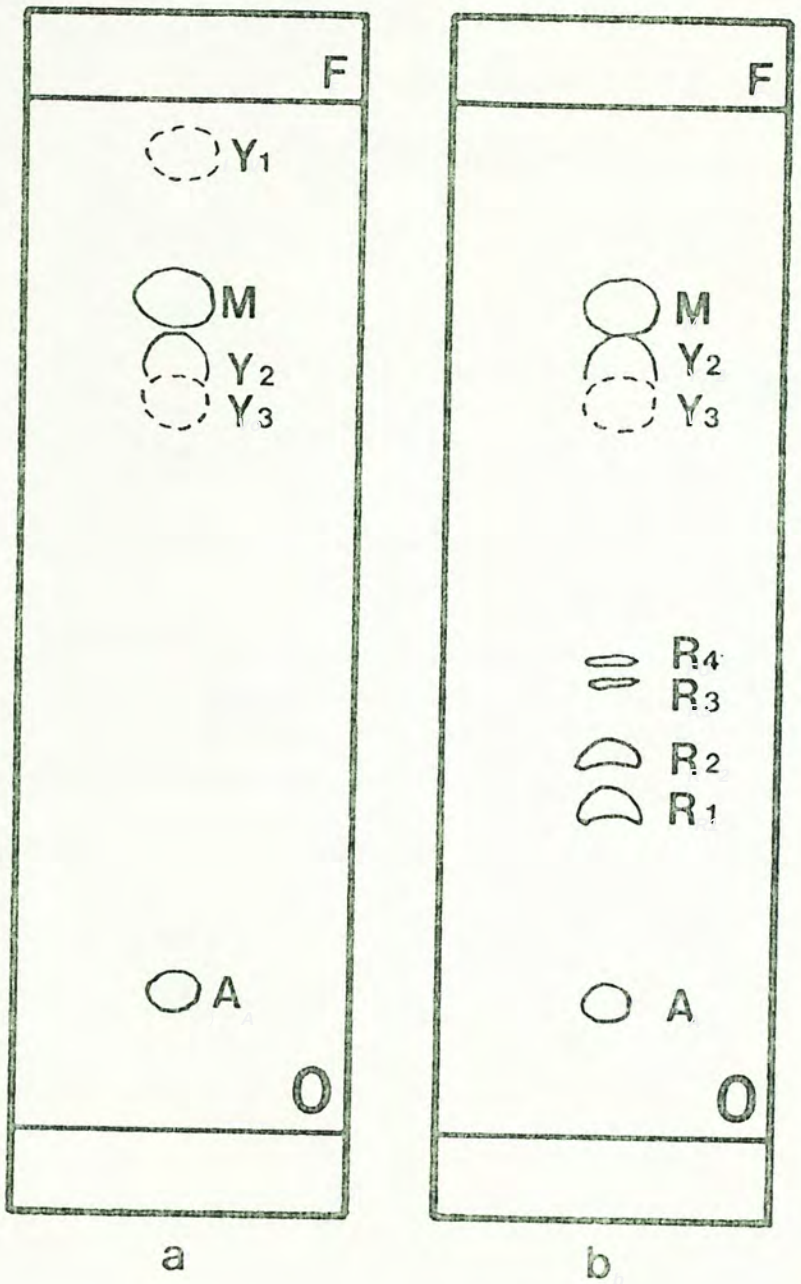


Fig. 6-1 : TLC of extracts of strain N11S developed with benzene:methanol:chloroform=30:10:9. a, extracted by petroleum ether; b, extracted by ethyl acetate. A, monascidin A; F, solvent front; M, yellow pigment; O, origin; R1, R2, R3, and R4, red pigments; Y1 and Y3, unknown compounds visible under UV light; Y2, unknown orange pigment.

creased with the age of colony. No anti-bacterial activity was detected from the young as well as old cultures of the lightly pigmented strains.

Table 6-2 : Rf values of pigments and antibiotic of Monascus purpureus by thin layer chromatography.

Compound	Color	Rf *	
		benzene:chloroform: methanol=83:12:3	benzene:methanol: chloroform=30:10:9
Monascin	Yellow	0.49	0.80
Monascorubrin			
R1	Red	0	0.33
R2	Red	0	0.38
R3	Red	0	0.45
R4	Red	0	0.47
Unknown			
Y1	Colorless	0.92	0.95
Y2	Orange	0.45	0.76
Y3	Colorless	0.22	0.73
Monascidin A	Pale yellow	0	0.14

*, The values are the means of ten determinations.

Fig. 6-2 : UV and visible absorption spectra of red pigments R1-R2 and R3-R4, and yellow pigment (M) in 95 % ethanol.

Fig. 6-3 : UV and visible absorption spectra of unknown compounds, Y1 and Y3, in 95 % ethanol and orange pigment (Y2) in distilled water.

Fig. 6-4 : UV and visible absorption spectra of monascidin A in 95 % ethanol.

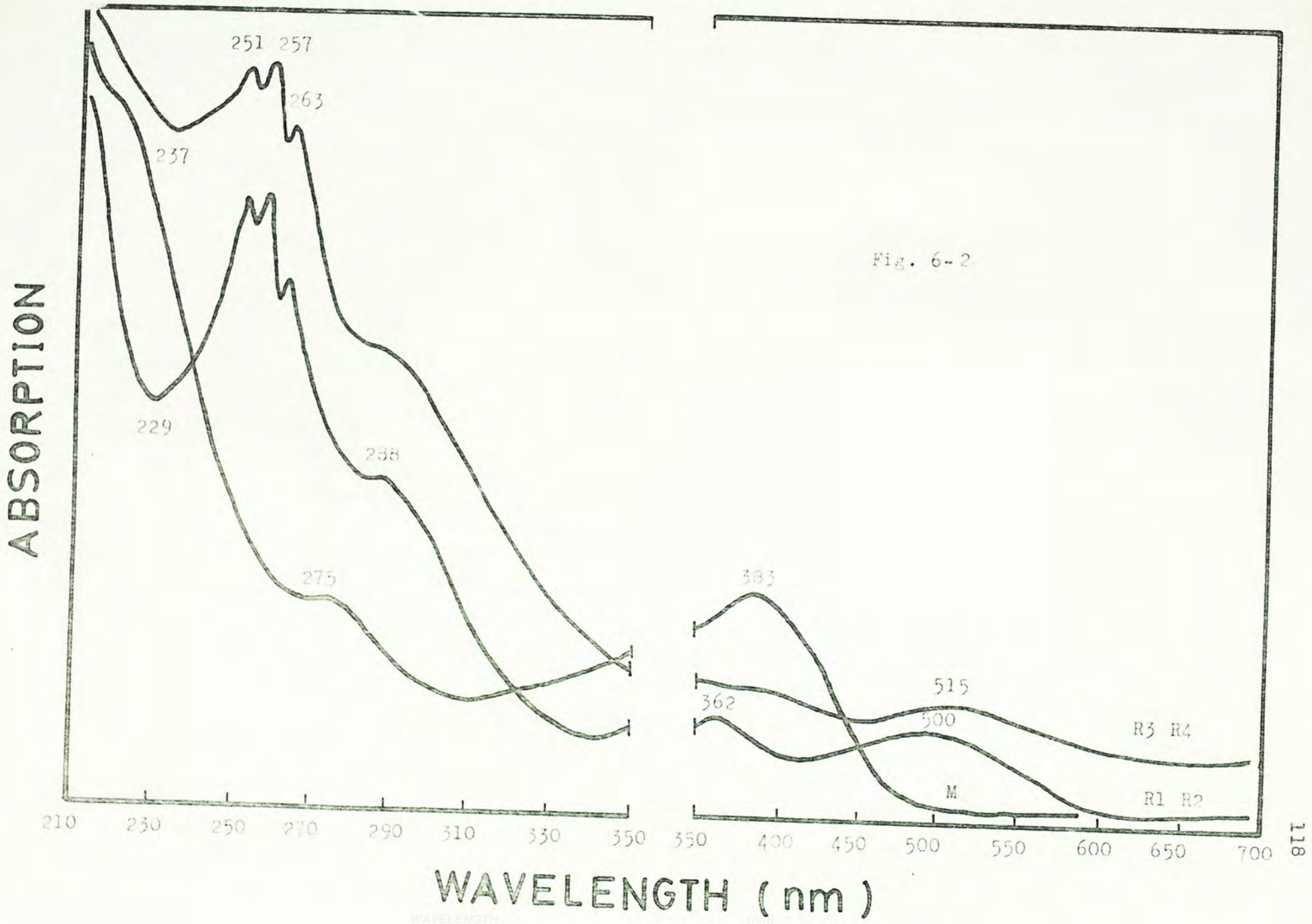

ABSORPTION
251 257
263
237
229
288
275
383
515
362
500
R3 R4
M
R1 R2
210 230 250 270 290 310 330 350
350 400 450 500 550 600 650 700
WAVELENGTH (nm)

Fig. 6-2

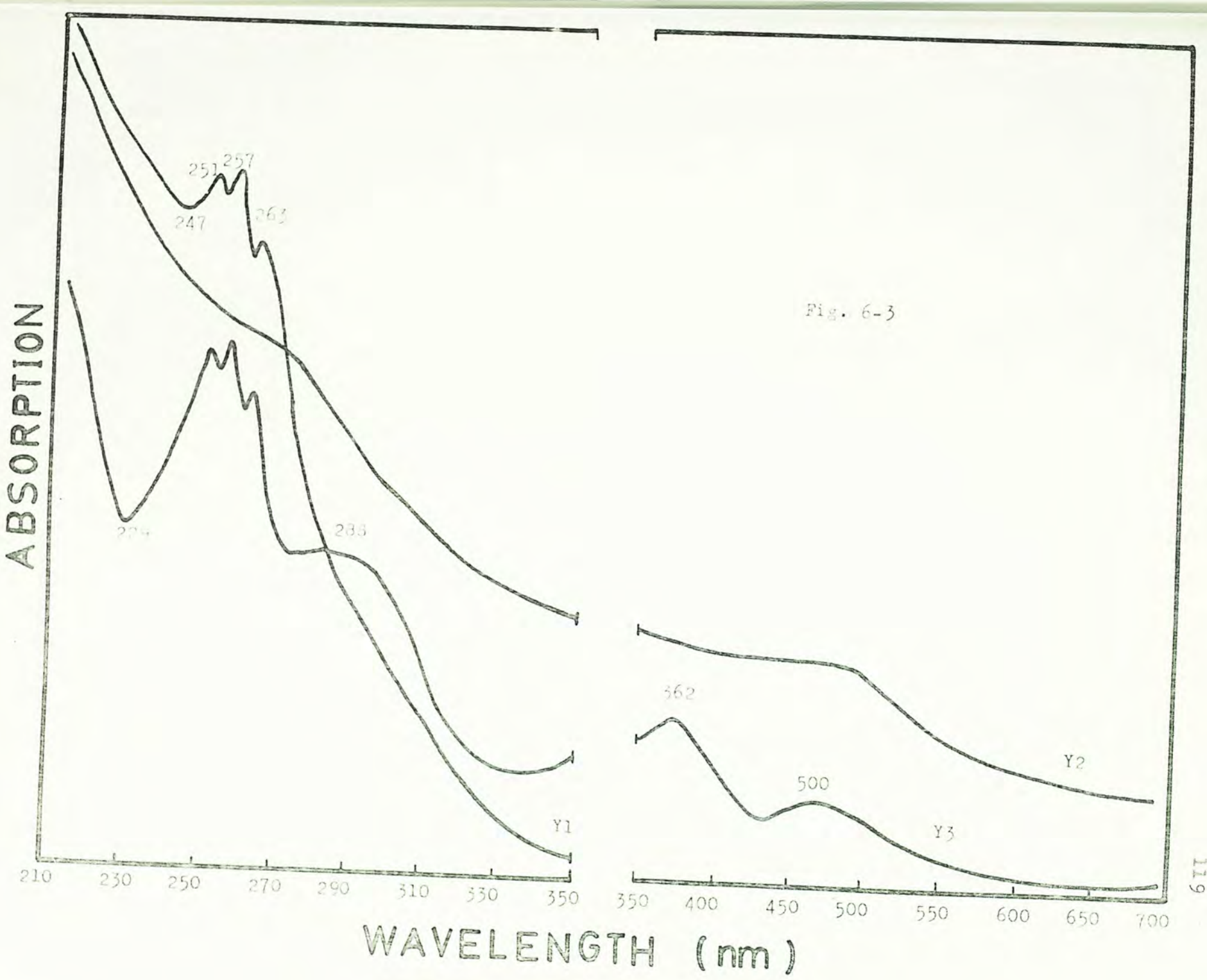

ABSORPTION
251
257
247
263
288
Y1
362
Y2
500
Y3
210
230
250
270
290
310
330
350
400
450
500
550
600
650
700
WAVELENGTH (nm)

Fig. 6-3

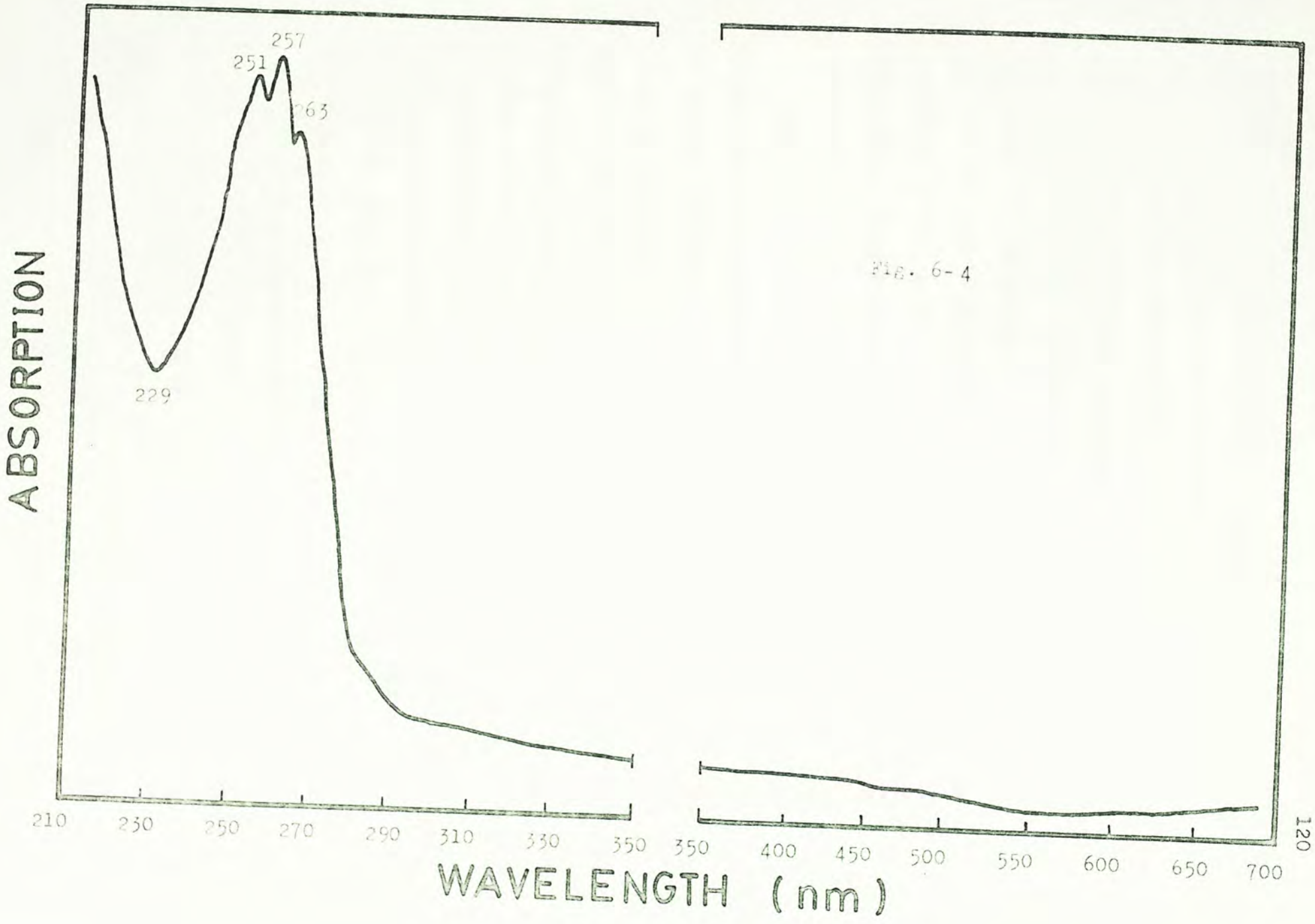

257
251
263
229
ABSORPTION
210
230
250
270
290
310
330
350
350
400
450
500
550
600
650
700
120
WAVELENGTH (nm)

FIG. 6-4

The anti-bacterial activity of Monascus purpureus in general was quite intense. The agar blocks cut from the vicinity of the day 10 N11S colony inhibited all the Bacillus species tested as well as Streptococcus, and Pseudomonas. It was inactive to Alcaligenes, Sarcina, Staphylococcus, Aerobacter, Escherichia, Salmonella, and Shigella. The inhibition zones varied in size with different species of Bacillus. Bacillus sp. (Plate 6-3) and B. megaterium de Bary were extremely sensitive to the antibiotic (Table 6-4). Monascidin was proposed as the name for the active compound(s).

The solvent system used by Chen et al. (Chen et al., 1971) in isolating monascin from Monascus anka Nakazawa et Sato was not suitable in separating the pigments and the anti-bacterial compounds in this study because the major anti-bacterial activity stayed at the origin in TLC (Table 6-2). Using an extremely polar solvent, such as n-butanol:pyridin:water=6:4:3, the anti-bacterial compound and the pigments moved near the solvent front in both TLC and paper chromatography. An intermediate polar solvent, benzene: methanol:chloroform=30:10:9, was used which had satisfactory resolving ability (Fig. 6-1).

The major active compound (A) was scratched from the TLC and dissolved in 95 % ethanol. Since it was pale yellow in color, the visible and the UV absorption spectra were determined. As shown in Fig. 6-4, there were no maxima in the visible range. The UV absorption spectrum of this compound resembled the red pigment, although there was no minor maximum or shoulder at 288 nm. Some inhibitory

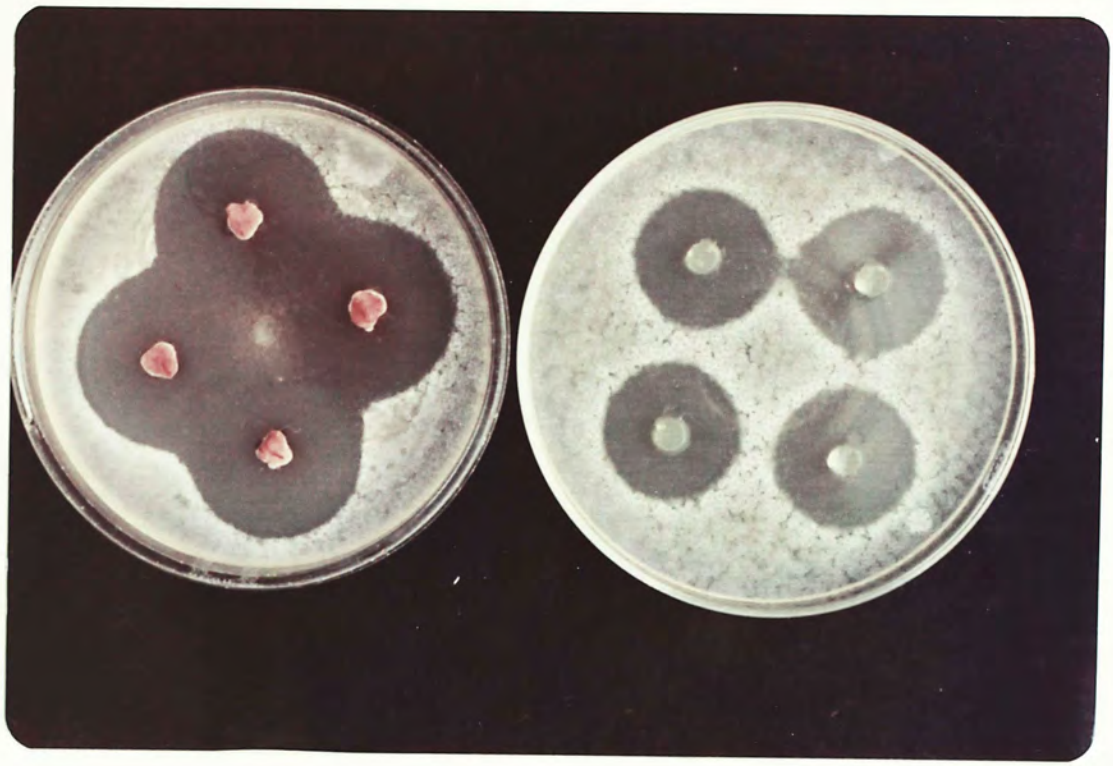


Plate 6-3 : Bioassay of anti-bacterial activity of M. purpureus. The indicator bacterium was Bacillus sp. The plate on the right shows inhibition zones exhibited by the agar blocks from the vicinity of 10-day cultures of N11S, while the left one contained mycelium pads cut from the center of the 10-day culture.

Table 6-3 : Comparative anti-bacterial activity of various strains of Monascus purpureus against Bacillus sp.

Strains	Diameter of inhibition zone (cm)*
Wild-type	1.59±0.07
N4S	1.39±0.10
N11S	2.36±0.18
N14S	0
X2P	1.88±0.23
X3S	0
X4P	0
X5S	0
L2S	1.96±0.25

*, Agar block of 0.6 cm in diameter and 0.3 cm thick was cut from the vicinity of 10-day colony and placed on the surface of soft agar inoculated with Bacillus sp., and the values are the means of ten determinations.

activity of compounds Y2 and Y3 were also detected, but these were negligible when compared to compound A. Paper chromatograms of the petroleum ether fraction developed with the TLC solvent, benzene: methanol:chloroform=30:10:9, showed three distinct action centers in bioassay. The Rf values of these action centers were about 0.3,

Table 6-4 : Action spectrum of Monascus purpureus N11S.

Bacteria *	Gram's stain	Diameter of inhibition zone (cm) **
Aerobacter aerogenes	-	0
Alcaligenes viscolact	-	0
Bacillus sp.	+	2.36±0.18
B. cereus	+	1.24±0.08
B. megaterium	+	2.27±0.13
B. sphaericus	+	1.23±0.10
B. subtilis	+	1.51±0.09
Escherichia coli B	-	0
E. coli poly A^+	-	0
Pseudomonas eisenbergii	-	1.14±0.20
P. fluorescens	-	1.00±0.00
Salmonella typhi	-	0
Sarcina lutea	+	0
Shigella sonnei	-	0
Staphylococcus aureus	+	0
Streptococcus aureus	+	1.16±0.09

*, Bacillus sp. was isolated from air-borne bacteria in the laboratory; Escherichia coli poly A^+ was furnished by Prof. S.C. Sheen, University of Kentucky, U. S. A.; Salmonella typhi and Shigella

sonnei were furnished by Prof. C.T. Huang, Hong Kong University; and the others were furnished by Dr. K.Y. Chen of The Chinese University of Hong Kong.

**, Agar blocks from the vicinity of 10-day N11S culture were of 0.3 cm thick and 0.6 cm in diameter.

0.6, and 1. Thus the antibiotic monascidin may consist of more than one compound.

Discussion

The various strains induced by fast-neutron, X-ray and laser radiations vary in pigmentation, macro- and micromorphology, and reproduction. Strains N4S, N11S and L2S are useful in the food industry since they produce more pigment than wild-type. Su et al. (Su et al., 1973) also isolated a pigment-enriched mutant by UV irradiation of Monascus anka which has been used in preparing red-mold rice and red rice wine in Taiwan.

In this study, the red pigment was further resolved into two groups, R1-R2 and R3-R4. The visible and the UV absorption spectra of fractions R1 and R2 were identical as were the spectra from R3 and R4. The differences between the spectra from these two groups of pigments were quite significant. It is believed that these two groups represent two different structures. According to Fielding et al. (Fielding et al., 1960), monascorubrin is usually mixed with

rubropunctatin, but the latter is orange colored (Haws et al, 1959). R1, R2, R3 and R4 are apparently not rubropunctatin.

Rubropunctatin can be oxidized to form monascin (Chen et al., 1971). The unknown orange pigment, Y2, from N11S and L2S reacted easily with alcohols to form red pigments. This orange compound also was not rubropunctatin, because monascorubrin has a longer saturated side chain than rubropunctatin (Fielding et al., 1960) and its interchange is not easy. Y2 could be a new compound closely related to the red pigment. It may be a metabolic intermediate which could provide clues about the biosynthesis of red pigments. The orange pigment(Y2) was partially bleached in air at room temperature overnight. A pigment extracted from M. anka was bleached by light (Su et al., 1973).

A number of fungi are already known to produce antibiotics (Broadbent, 1966). The antibiotic activity of different fungi used in oriental food has been investigated (Wang et al., 1972), but the anti-bacterial activity of Monascus, a fungus used in Chinese medicine has been neglected. Monascidin is a potent but not a broad spectrum antibiotic. It is effective to all Bacillus species tested especially to Bacillus sp. and B. megaterium. Though the red-mold rice has been used as the main constituent for fever curing, monascidin did not inhibit the local fever pathogens, Salmonella typhi Warren et Scott and Shigella sonnei (Levine) Weldin. Red-mold rice, therefore may not directly act on the pathogens.

The production of antibiotics and pigments from the strains were regulated by light. Though N14S was albino and inactive to

bacteria, pigments and antibiotics were synthesized after UV and continuous white light irradiations (Chapter Five). It is reasonable to propose that monascidin is synthesized in the same pathway as the pigments.

The anti-bacterial activity of monascidin may be valuable commercially.

CHAPTER SEVEN

CONCLUSION

Since the first description of Monascus purpureus Went in 1895, only a few papers about this fungus have been published. Besides the morphological studies in the decade following its discovery, the main interest of recent investigations is on two schemes. One is the studies on the enzyme productions of this fungus by a number of biologists mostly in Japan (Enzyme structure and function research group, 1976; Kitahara and Murata, 1954; Saito, 1925; Saruno et al., 1964; Yamada, 1957) and furthermore to its application in food industry (Ho et al., 1973; Palo et al., 1960; Su et al., 1970 and 1973). The other is of its pigments accomplished primarily from some groups of English chemists (Chen et al., 1971; Fielding et al., 1960 and 1961; Hadfield et al., 1967; Haws and Holker, 1961; Haws et al., 1959; Kurono et al., 1963; Nishikawa, 1932). Of course, there are still some others who are interested in the mycological aspects, as already mentioned in Chapter One.

In this study, some related aspects of the fungus were investigated and the results would encourage further endeavours. A brief conclusion about this study and some prospects are given in this chapter.

M. purpureus produces both conidium and ascospore. On malt extract agar medium in slant or in petri dish, this fungus produced much more ascospores from day 4 and since then. But in the liquid

medium of the same composition excluding agar only, shaking at 200 rpm, the culture contained numerous conidia by same growth stage. So, aeration might play an important part in the production of spore kinds. Good aeration was also necessary for the general growth of this fungus, because plugging of the petri dish edges by water droplets could greatly inhibit the growth of mycelium.

The ontogeny of cleistothecium and ascospore of M. purpureus might need reconsideration. The number of asci in each cleistothecium and the number of ascospore in each ascus of this fungus have not been determined. In the X-ray induced strain X2P, a single ascus with a few ascospores in one cleistothecium was observed. In the wild-type, generally over hundred of ascospores occur in each cleistothecium.

As revealed in Chapter Two, the physiology and morphology of conidium and ascospore germination are different. Conidium could utilize more carbon sources and required less exogenous nutrients, metallic salts and growth factors. The germination percentage of ascospore was much lower, but ascospore was more resistant to high temperature and fast-neutron irradiation. The red pigment existed on the wall of ascospore might be responsible for its resistance, just as melanin (Ellis and Griffiths, 1975) and carotenoids (Macmillan et al., 1966) in other species.

Light can also regulate the germination of fungal spores. Spore germination in Phytophthora heveae Thomps. is stimulated by blue light (Berg and Gallegly, 1966). In Puccinia species the uredospore germination is inhibited by blue and far-red irradiations (Calpouzos and Chang, 1971; Givan and Bromfield, 1964). A preliminary investigation

has been done and shown that blue, red and far-red irradiations were inactive to spore germination of Monascus purpureus, and UV light has not been tried. Since detailed investigation on this aspect has not been done, so it is not mentioned in Chapter Two.

Though most of the ionizing irradiations did not killed the conidia and ascospores of M. purpureus, they were quite efficient in inducing mutants. The strains obtained in this study differred in morphology that would probably reflect their different physiological activities. The fast growing ones were slightly colored while those had more or less the same growth rates as wild-type were deeply pigmented. The most exceptional feature was the production of conidia in great numbers for some strains. The ascospore production were inhibited in all the strains.

The strains which might probably have genetic changes provide favorable materials for the study of morphogenesis. Indeed, the photoresponses and pigmentation of these strains have been investigated as reported in Chapters Five and Six. Wild-type as well as all the induced strains were photoresponding. The main phenomena of light effect were the stimulated pigmentation and conidium production, and the latter one was less prominant. Those slightly pigmented strains responded sensitively to UV light. The pigmentation process might possibly be blocked by the ionizing irradiation and it could be restored or released to some extent by the stimulation of UV light. Blue light which is stimulating to carotenoid synthesis in other fungus (Fabo et al., 1976) was inactive to the strains of M. purpureus. The substitution of light irradiation by unknown compound(s) excreted by

Bacillus sp. to stimulate pigmentation also provides clues for the photochemical reactions taken place in the photoresponses.

Monascus purpureus was known to produce anti-bacterial compound(s) named monascidin. The major active compound, monascidin A, was a pale yellow one which had UV absorption spectrum similar to the red pigments. The molecular structure of monascidin A has not been determined. It is also necessary to have a detailed screening of the antibiotic activity of this fungus. It was intensively inhibitory to *Bacillus* species among the few genera studied.

The production of antibiotic in those slightly pigmented strains was also stimulated by light. Thus, a further study on photoresponses should include both the pigmentation and antibiotic production.

SUMMARY

Though Monascus purpureus Went was first described in 1895 in Java, this species was originally grown in China. The fungus is known to the Western world as a contaminant on cereals, starch and silage. In China, Japan, Indonesia and other oriental countries, this fungus is used to prepare red-mold rice, red bean curd and red rice wine. It is also used as a food disinfectant and a natural food coloring matter. In traditional Chinese medicine, this fungus has been widely used for hundreds of years to treat several diseases.

This study included the spore germination, effects of ionizing irradiations, morphology of induced strains, photoresponses, pigmentation, and anti-bacterial activity of M. purpureus.

The germination of conidium and of ascospore differred in morphology and physiology. As a whole, conidium was rich in energe storage requiring less exogenous carbon sources and growth factors. But ascospore was more resistant to high temperature as well as ionizing irradiations.

Though the conidium and the ascospore were not killed by most irradiation treatments, strains of special interests were induced. The most exceptional feature was the reversed mode of spore production. Instead of producing ascospore, numerous conidia were produced by most strains and the ascospore production of all the strains was inhibited. They also differred in the hyphal texture, pigmentation and anti-bacterial activity.

The slightly colored strains were extremely sensitive to UV

light. The most prominant effects of UV irradiation were stimulated pigmentation and conidiation. An unknown compound(s) excreted by Bacillus sp. could substitute the UV irradiation to stimulate pigmentation.

Monascus purpureus produced anti-bacterial compound(s) designated to be monascidin, which was active to Bacillus, Pseudomonas and Streptococcus and the activity was quite intense. A major active compound, monascidin A, was pale yellow colored and had UV absorption spectrum similar to that of red pigments produced by the wild-type and all other strains.

物理因子對紅麴菌生長的影響

摘 要

紅麴菌原產中國。在日本、印尼、菲律賓等東亞地區，多具經濟價值。可製紅麴、南乳、紅老酒等，亦可作食物防腐劑和染料。入藥可治痢疾、跌打損傷、飲食停滯等症。

本研究包括孢子發芽、電離輻射影響、新品系的誘導和形態、照光反應、色素和抗生素生成等。

分生孢子和子囊孢子的發芽在形態上和生理上均有差異。總括來說，分生孢子發芽較快及具極高的發芽率，並需較少外在營養。但是子囊孢子則具抗高熱與電離輻射的能力。

在野生種，子囊孢子佔絕大多數，但在新品系中多產生大量的分生孢子。其他的相異點有菌絲的性質、色素和抗細菌能力等。

微具色素的新品系對紫外光照射都極敏感，且能刺激色素和分生孢子的生成。

紅麴產生的抗生素是為紅麴素，對桿菌、假單孢菌和鏈菌等屬的細菌有強烈的抑制作用。其中再分離出淺黃色主要成分，稱為紅麴素甲。該化合物的紫外光吸收光譜和紅麴菌的紅色素相類似。

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